Reply Under 37 C.F.R. §1.116
Expedited Procedure
Group Art Unit 1617

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION OF	Confirmation No.: 5040
Engel *et al.*	Group Art Unit: 1617
Appln. No.: 09/523,455	Examiner: S.A. Jiang
Filed: March 10, 2000	

Title: Method for a Programmed Controlled Ovarian Stimulation Protocol

December 13, 2005

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DECLARATION BY DR. RIETHMÜLLER-WINZEN, M.D., PURSUANT TO 37 C.F.R. §1.132

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Hilde Riethmuller-Winzen declare as follows that:

1. I am the chief executive officer of the Dr. Riethmüller M/R/S GmbH providing medical research services to Zentaris GmbH amongst other pharmaceutical companies. I earned a doctorate in medicine at the Justus-Liebig-University in Giessen, Germany in 1982. A copy of my curriculum vitae is attached (see Appendix A).

2. I have worked in the field of clinical pharmacology and embryology with a focus on development of drugs and clinical programs for human reproduction for the past 11 years.

3. My office is currently located at Dr. Riethmüller M/R/S GmbH, Mittelweg 27, 60318 Frankfurt, Germany.

4. I am a named co-inventor of U.S. Patent Application No. 09/523,455.

5. I have reviewed the official action dated October 4, 2004 and June 13, 2005, issued in connection with U.S. Patent Application No. 09/523,455.

6. I have also reviewed each of the references cited by the examiner, *i.e.*, Engel *et al.* (EP 0788 799; hereafter "Engel"), Albano *et al.* (*Hum. Reprod.* 11:2114-2118 (1996); hereafter "Albano"), Felberbaum *et al.* (10th World Congress on In Vitro Ferlization and Assisted Reproduction, Gomel *et al.* (Eds.), Moduzzi Editore, Bologna, Italy pgs. 397-404 (1997); hereafter "Felberbaum"), and Garfield *et al.* (U.S. Patent No. 5,470,847; hereafter Garfield) in view of Deghenghi (U.S. Patent No. 5,945,128; hereafter "Deghenghi"), Rabasseda *et al.* (Drugs of the Future, 24:393-403 (1999); hereafter "Rabasseda"), and Kent (U.S. Patent No. 4,016,259; hereafter "Kent").

7. I make this declaration in response to the official actions dated October 4, 2003 and June 13, 2005, in which currently pending claims 1 and 4-24 were rejected as allegedly being obvious over Engel, Albano, Felberbaum, and Garfield in view of Deghenghi, Rabasseda, and Kent. It is my understanding that the examiner alleged that one having ordinary skill would have been motivated to employ the particular LHRH-antagonist such as teverelix, antide, and abarelix in methods of controlled ovarian stimulation and assisted reproductive techniques according to Engel, Albano, Felberbaum, Deghenghi and Rabasseda. The examiner alleged that the results in the instant method on pages 4 and 5 of the specification provide no clear and convincing evidence of non-obviousness or unexpected results over the cited prior art since there is no side-by-side comparison with the closest prior art. The examiner concluded that since all method and composition components herein are known to be useful to treat or manage the infertility, it is considered *prima facie* obvious to combine them into a single method useful for the very same purpose.

8. I disagree with the examiner's conclusion, that, based upon a review of the above-identified references, a scientist would successfully perform, or even hold a reasonable expectation of success in performing methods for treating or managing infertility via use of LHRH antagonists in conjunction with progesterone, or progesterone plus oral contraceptives in order to program controlled ovarian stimulation and assisted reproductive techniques by resetting the menstrual cycle.

9. I invented along with my colleague Dr. Jurgen Engel, a novel and unobvious method for therapeutic management of infertility and increasing the quality of fertilized oocytes and embryos by programming controlled ovarian stimulation (COS) and assisted reproductive techniques (ART) in order to optimize oocyte harvesting and fertilization during the clinical week of Mondays to Fridays, the method comprising the following steps: (a) programming the start of controlled ovarian stimulation by resetting the menstrual cycle through administration of a compound selected from the group consisting of an LHRH antagonists, a progestogen only preparation, a combined oral contraceptive preparation, and a combination thereof wherein the LHRH antagonist is selected from the group consisting of cetrorelix, teverelix, ganirelix, antide, and abavelix and is administered at a dosage range between 0.5 mg to 10 mg during the luteal phase of the menstrual cycle to induce luteolysis, and wherein the progestogen only preparations and/or the combined oral contraception preparations are administered starting during both the luteal phase and day 1 or 2 of the menstrual cycle; (b) exogenous stimulation of the ovarian follicle growth via administration of a compound selected from the group consisting of urinary FSH, recombinant FSH, HMG, recombinant LH, clomiphene, and a combination thereof; (c) suppression of premature ovulation via administration of an LHRH-antagonist selected from the group consisting of cetrorelix, teverelix, ganirelix, antide, and abavelix during the follicular stage of the menstrual cycle; (d) induce ovulation via administration of HCG; and (e) application of assisted reproduction techniques, especially IVF, ICSI, GIFT, ZIFT or by intrauterine insemination by sperm injection.

In contrast, the cited publications merely focus on preventing ovulation and LH surges, and fail to teach programming controlled ovarian stimulation or assisted reproductive techniques.

10. The evidence presented in the application also teaches that the injection of LHRH antagonists such as cetrorelix, teverelix, ganierelix, antide or abarelix is used in two novel ways. The first way is to use the LHRH antagonists for the purpose of programming the beginning of the menstrual cycle. This is accomplished by using the LHRH antagonist for ending the previous normal menstrual cycle via luteolysis, or a degeneration of the corpus leteum. We have also

demonstrated that progestogens (or the combination of progestogens and estradiol) can be used to reset the menstrual cycle. Once the menstrual cycle is reset, physicians will be able to control the timing of ovarian stimulation and fertilization of the eggs using assisted reproductive techniques.

The second use of LHRH antagonists is to prevent a premature luteinizing hormone (LH) surge and subsequent unexpected ovulation during the controlled ovarian stimulation and assisted reproductive technique step of the claimed method. Accordingly, we respectfully submit that the novel use of contraceptives such as progestogens (or the combination of progestogens and estradiol) in combination with LHRH antagonists are used to reset the menstrual cycle in order to readily program controlled ovarian stimulation. This method further allows physicians to predict the day in which to perform assisted reproductive techniques to maximize fertilizing an egg. The seven publication presented by the examiner neither teach nor suggest this claimed invention. Each of the references will be discussed and compared to our invention below.

11. Controlled ovarian stimulation for assisted reproductive techniques (COS/ART) represents the treatment of choice to overcome infertility. The intention of this method is to obtain supernumerary follicle and thus oocyte growth. The consequence of this method is that more than one embryo can be obtained and placed into the uterus of a woman. Thus, the possibility to become pregnant is tremendously increased by 25 to 40 percent. When applying treatment modalities to overcome infertility, the physiologic endocrine situation in women has to be respected and therapeutic approaches have to be adopted accordingly. Therefore, the physiology of the menstrual cycle dictates the methods and medications to be chosen. However, many feed-back mechanisms regulating normal and stimulated menstrual cycles are still not completely understood and require more research activities.

According to the state of the art, growth of multiples of follicles/oocytes can only be induced by the exogenous administration of compounds containing follicle stimulating hormones (FSH) or by stimulation of the estrogen receptor by antiestrogens with proestrogenic components, such as clomiphene. Estradiol synthesis is induced in each of the growing follicles by the secretion of follicle stimulating hormone (FSH) from the pituitary or by exogenous administration. Due

to the development of many follicles/oocytes, high estradiol levels occur at an early stage of the cycle. In a normal menstrual cycle, high estradiol concentrations signalize that the oocyte is mature and can thus be released from the follicle to be fertilized. Therefore, a high amount of LH is released from the pituitary to induce the rupture of the follicle and to release the oocyte. However, when high estradiol levels in a FSH-stimulated cycle are measured, the oocytes are still immature and the LH surge has to be prevented. This is only possible by LHRH analogs (antagonists or agonists) competing with natural luteinizing hormone releasing hormone (LHRH) at the receptors of the pituitary.

12. The oocytes obtained at the day of their release from the follicles (*i.e.*, ovarian stimulation) are of different sizes and grade of maturity. The different grade of maturity hampers the development of good quality embryos. A goal of COS/ART is to obtain follicles/oocytes of optimal and synchronous size and maturity.

Not only are good quality oocytes required, but the number of oocytes released is important. The uncontrolled release of the oocytes *via* premature LH surges from the follicles at a unpredictable time is also not recommended as this would induce the implantation of many embryos and thus endangers the life of the female patient. Premature LH surges at the state of immaturity of the oocytes occur only in about 50% of the patients undergoing ovarian stimulation (OS). It is not known why premature LH surges are not induced in all ovarian stimulation cycles.

To solve both issues related to oocyte stimulation, the claimed method requires that the release of the oocytes from the follicles is triggered by human chorionic gonadotropin (HCG) at the state of maturity of the majority of the oocytes. Oocytes can be recovered surgically (OPU) and then be fertilized in the laboratory. After two or five days, one to three viable embryos can be transferred (ET) into the uterus to generate pregnancy. Supernumerary embryos can be frozen and replaced lateron in another menstrual cycle.

13. Engel, Albano, Felberbaum, and Rabasseda disclose the normal ovarian stimulation procedure that requires prevention of a premature LH surge carried out with an LHRH antagonist and induction of ovulation by a suitable

compound, *e.g.*, HCG. Engel, Albano, Felberbaum, and Rabasseda disclose the use of an LHRH antagonist **only** during the follicular phase (*i.e.* cycle day 1 until day of ovulation) for purposes of controlling ovarian stimulation, **but do not report the application of an LHRH antagonist during the luteal phase** (*i.e.*, day of ovulation until end of menstrual cycle) or for programming or resetting a menstrual cycle for purposes of controlling the exact day of ovarian stimulation and assisted reproductive techniques (*emphasis added*).

14. Although the effective amount of LHRH antagonist reported by Engel, Albano, Felberbaum, and Rabasseda are comparable to the amounts disclosed in the application for ovarian stimulation, none of these references teach administering LHRH antagonists or progestogens or contraceptive preparations during the luteal phase to reset the menstrual cycle for purposes of programming oocyte pick-up and embryo transfer in the following cycle.

15. The aim of the LHRH administration by Engel, Albano, Felberbaum, and Rabasseda is to prevent a premature LH surge during ovarian stimulation of the follicular phase.

In contrast, our invention calls for administration of progestogens, contraceptive preparations or LHRH antagonists during the luteal phase to reset the menstrual cycle, which further enables physicians to schedule more effective oocyte pick-up and embryo transfer in the following cycle. More importantly, our method has been shown to cause unexpected homogenization of follicle size before ovarian stimulation occurs (*see* Fanchin *et al.*, *Reprod. Biomed. Online* 10:721-728 (2005); hereafter Fanchin; Appendix B). Accordingly, our method of using several compounds (LHRH antagonist, progestogen only or a combined oral contraceptive preparation) for resetting the menstrual cycle is neither taught nor suggested by Engel, Albano, and Felberbaum either alone or in combination.

16. Specifically, Engel teaches administration of progesterone only after ovarian stimulation to support a fertilized oocyte at the beginning of pregnancy. Engel does not teach or suggest the use of progesterone to program a menstrual cycle,

i.e., to manipulate, prolong or interrupt a menstrual cycle in order to initiate a new menstrual cycle to predetermine ART procedures.

17. Albano reports administering LHRH antagonists during the follicular phase to decrease progesterone concentrations to a mean of 0.27 μg/L to 0.12 μg/L. Albano, however, does not teach the use of progesterone in the reduction of high luteal phase progesterone values that peak at about 25 mg/day 6 to 8 days after the LH surge in a normal menstrual cycle (*see* The Merck Manual of Diagnosis and Therapy, 17th Edition. Editors Beers MH and R. Berkow, Section 18, page 6, 3rd full paragraph; Appendix C)) or in the programming of a new menstrual cycle to calculate the initiation of OS and performance of ART procedures on working days.

18. Felberbaum does not teach the fall of sex steroids due to the administration of LHRH antagonists but of LHRH agonists (page 398, last paragraph), *i.e.*, of a class of drugs with a completely different mechanism of action compared to LHRH antagonists. Additionally, Feleberbaum does not report the use of LHRH antagonists, progestogen, or contraceptive preparations for resetting the menstrual cycle and programming the COS and ART procedures.

19. Garfield also does not teach the use of LHRH antagonists, progestogen, or contraceptive preparations for resetting the menstrual cycle to program COS and ART procedures. Garfield describes extensively physiologic and endocrine conditions and feed-back mechanisms (*see* col. 2 lines 9-17) as well as substances and procedures for the prevention of conception or ovulation in a physiologic non-manipulated menstrual cycle, *i.e.*, for the prevention of the implantation of a viable embryo and thus of pregnancy (*see* col. 1 lines 9-17).

This, however, is not the target of our invention. Our invention does not use progesterone alone or with any other compound **for the inhibition of ovulation as reported by Garfield** in col. 5 lines 35-38. Additionally, Garfield does not teach the use of any substance for the resetting of the menstrual cycle for purposes of programming the COS and ART procedures or for ovarian stimulation.

20. Deghenghi discloses the process for manufacturing a pharmaceutical composition for the delivery of an effective amount of several LHRH antagonists amongst other bioactive peptides or peptide analogs (col. 2 lines 19-23), but does not teach the use of progesterone, LHRH antagonists, or contraceptive preparations for the programming of COS and ART procedures.

21. Kent discloses the combination of progestogens and estrogens in contraception (col. 1 lines 20-25), but does not teach the use of progesterone, LHRH antagonists, or contraceptive preparations for resetting the menstrual cycle in order to program COS and ART procedures for increasing changes of pregnancy.

22. **Accordingly, the use of LHRH antagonists, progestogen or contraceptive preparations to program COS and ART has not been reported by any of the references.** As has been shown by Fanchin (Appendix B), another benefit of programming the COS/ART procedures through resetting the menstrual cycle is to homogenize the antral follicle sizes that are often markedly heterogeneous during the early follicular phase. Use of a method defined by Engel, Albano, Felberbaum, Garfield in view of Deghengi, Kent, and Rabasseda, would cause follicle size discrepancies to occur during the coordinated follicular growth of ovarian stimulation. The cited prior arts' oocyte stimulation methods thereby reduce the number of follicles that reach maturation simultaneously.

Fanchin (Appendix B) teaches the applicants' method of suppressing luteal FSH concentrations of the menstrual cycle preceding the COS and ART cycle by the administration of either oral estradiol preparations or the injection of an LHRH antagonist during the luteal phase. The results showed that luteal FSH suppression by either estradiol or LHRH antagonist administration reduced the size and improved the homogeneity of early antral follicles during the early follicular phase, an effect that persisted during ovarian stimulation. The investigators concluded that coordination of follicular development via luteal FSH suppression may optimize ovarian response to short LHRH antagonist protocols, and also constitute an attractive approach to improve the outcome of ovarian stimulation procedures.

23. The need for homogenizing the antral follicle sizes is also summarized by Hugues, "Medical, Ethical and Social Aspects of Assisted Reproduction" held at WHO Headquarters in Geneva, Switzerland (Appendix D) on COS/ART procedures. In Hugues, the investigator stated that "furthermore, programming the IVF cycle through steroid administration during the cycle preceding IVF may not only be convenient for most centres, but could also be effective in improving the size of the cohort of recruited follicles." (*see* page 105, second column) The benefit of a programming cycle is currently under consideration (*see* page 105 of Hugues). Accordingly, both Fanchin and Hugues demonstrate the importance of resetting the menstrual cycle and programming COS/ART procedures for increasing the quality of fertilized oocytes and embryos.

24. From the investigations carried out by Engel, Albano, Felberbaum, and Garfield in view of Rabasseda, Deghengi, and Kent, one of ordinary skill in the art would not have been motivated to employ the programming of COS and ART procedures by the use of LHRH antagonists, progestogen, or contraceptive preparations. The critical difference between the claimed invention and the cited prior art is the injection of an LHRH antagonist during the luteal phase of the menstrual cycle **preceding the cycle** of COS and ART procedures. One of skill reviewing the cited art would understand the risk associated with administration of LHRH antagonist during the luteal phase. This risk is the destruction of the follicles because of the abrupt decrease in LH and FSH concentrations during the luteal phase. One of skill would actually expect only a limited number of follicles/oocytes to develop and be obtained for fertilization or the protocols discussed in Engel, Albano, Felberbaum, and Garfield. Furthermore, one of skill would perceive these risks to produce heterogeneous follicles at the beginning of the ovarian stimulation cycle. Accordingly, our invention unexpectedly overcomes these risks to produce follicles of more homogenous sizes in a predictable program for a patient.

25. With regard to programming the ovarian stimulation cycle by progestogens or contraceptive preparations, the risk exists that high concentrations of

progesterone still available at the beginning of ovarian stimulation endanger the development and quality of follicles/oocytes during the first days of ovarian stimulation before an LHRH antagonist is given for the prevention of a premature LH surge. Furthermore, an overhang of progestogen or estradiol forming parts of the contraceptive preparation could negatively influence the secretion of FSH from the pituitary due to feedback with the LHRH receptors. In consequence, the development of follicles/oocytes could be disturbed. (See Figure 1)

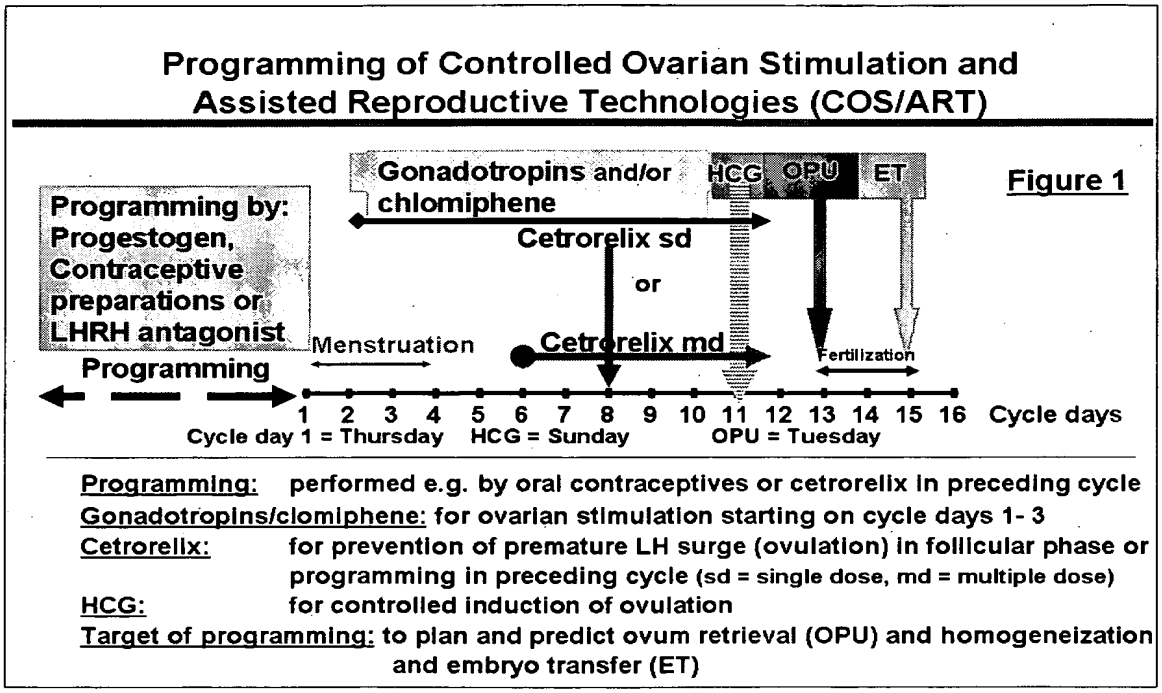

Figure 1: Programming of ovarian stimulation and ART with progesterone, contraceptive preparations or LHRH antagonists

26. Furthermore and in contrast to Garfield, administration of contraceptive preparations for prevention of ovulation in a COS/ART cycle is not practicable as **progesterone** levels would be elevated during ovarian stimulation due to the administration of progesterone/progestogen found in contraceptives. The co-administration of drugs for programming together with gonadotropins and LHRH LHRH antagonists would have deleterious effects on the (1) growth and development of follicles, (2) oocyte maturation due to complex hormonal feed-back mechanisms, and (3) direct effects on the follicles and endometrium. This is in stark to Albano.

Albano teaches lowering progesterone levels as low as possible during the follicular phase. Accordingly, the cited art doesn't teach or suggest that progesterone/progestogen contraceptives may be used to program COS/ART procedures even though their use occurs during the 1st days of the menstrual cycle.

27. In summary, a side-by-side comparison of the seven publications presented by the examiner with the present invention disclosed by the applicants show the following:

(a) The target of administration of contraceptive preparations or progestogens as administered by the applicants is the programming of a new menstrual cycle and thus of COS/ART, but not the prevention of ovulation and conception as presented in the cited prior art (Engel, Albano, Felberbaum, and Garfield in view of Deghenghi, Rabasseda, and Kent).

(b) Injection of LHRH antagonists, preferably cetrorelix, as administered by the applicants fulfills two objectives within two different menstrual cycles:

(i) Luteolysis of the previous 'normal' menstrual cycle and thus programming/timing of the COS/ART procedures is novel and has not been reported by any of the references; and

(ii) the administration of progestogen/contraceptive preparations and/or an LHRH antagonist during the luteal phase of the menstrual cycle preceding COS/ART procedures is convenient for most fertility centers, but could also be effective in improving the size of the cohort of recruited follicles and thus further improve the outcome of ovarian stimulation procedures.

c) The underlying invention does not combine known compositions useful in the therapeutic management of infertility. The invention discloses the novel use of contraceptives, *i.e.* progestogens or the combination of progestogens and of estradiol, or the use of LHRH antagonists in the programming of COS/ART protocols and in improving the homogeneity and quality of the follicles/oocytes for embryonic formation. Therefore, it is my opinion that the present invention is not obvious in view of the cited prior art.

28. All statements made herein of my own knowledge are true and that all statements made on information and believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under section 101 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

12/13/2005

Dr. Hilde Riethmüller-Winzen Date

CURRICULUM VITAE

PERSONAL DATA

Name:	**Dr. med. HILDE RIETHMÜLLER-WINZEN**
Date of birth:	July 11, 1951
Nationality	German
Languages:	German, English, Spanish

EDUCATION

1970 - 1976	Study of Human Medicine at the Justus-Liebig-University (JLU) Giessen
1977	Medical Licensing (Approbation)
1982	Promotion to Dr. med. (JLU Giessen)
1982	Recognition as Physician Specialised in Anesthesiology and Intensive Care (General Medical Council of Hesse)
1984, 1988	Registration into the Medical Register (Alliance of Panel Doctors of Nordrhein and Hesse)

PROFESSIONAL CAREER

1976 - 1977	Medical Internship in Surgery and Internal Medicine at the JLU Giessen
1977 - 1982	Scientific Assistant at the Dept. of Anesthesiology, Surgical Intensive Care and Outpatient Pain Management (Director: Prof. Dr. G. Hempelmann, Prof. Dr. H.F. Herget).
1982	Senior Physician Specialised in Anesthesiology at the Dormagen County Hospital, NRW (Director: Prof. Dr. H. Siepmann).
1983	Senior Physician Specialised in Anesthesiology at Cologne, Köln-Merheim Municipal Hospital (Director: Prof. Dr. G. Matthes).
1982 - 1984	Deputize for General Practitioners occasionally for several months to obtain Registration into the Medical Register.
1984	Medical Scientist at Troponwerke, Cologne (Bayer AG), for the performance of Phase I - III trials of research or commercialised compounds (cardiologic, antirheumatic and CNS-acting drugs).
1985	Head of Department Medical Research Analgesics of ASTA Media AG (pharmaceutical section of Degussa AG), Frankfurt; development and registration of new analgesics.

1986	Head of Department Clinical Pharmacology of ASTA Medica AG.
1991	Member of Board of Directors and Head of Medical Affairs (Medical Director) of ASTA Medica S.A. Madrid, Spain (previously: Laboratories Sarget S. A.).
1994	Head of Medical Research Endocrinology of ASTA Medica AG, Frankfurt; development and registration of endocrinologic new chemical entities in benign and oncological indications.
2001	Establishment and CEO of AGOM GmbH, DR. RIETHMÜLLER M/R/S GmbH (since 2002); medical consultant for clinical development and registration of drugs and medical devices.

MEMBERSHIPS

- Professional Association of German Anesthesiologists
- German Society for Pharmacology and Toxicology
- Society for Clinical Pharmacology e. V.
- Association for Applied Human Pharmacology e.V.
- Alliance of Panel Doctors, Hesse
- European Society for Human Reproduction and Embryology (ESHRE).

SCIENTIFIC ACTIVITIES

- Performance of Phase I - IV trials since 1978
- Publications and meeting papers
- Congress presentations

International reports and expert opinions for the submission of the application of new drug entities for marketing authorization approval.

Frankfurt, May 2004
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Outlook

Hormonal manipulations in the luteal phase to coordinate subsequent antral follicle growth during ovarian stimulation

Renato Fanchin is a gynaecologist, PhD, specializing in Reproductive Medicine. He is currently the Chief of Division of Reproductive Medicine in the Department of Obstetrics and Gynecology and Reproductive Medicine at the Hôpital Antoine Béclère, Clamart, France. His scientific centres of interest are the identification of clinical markers of ovarian function, the development of new approaches of ovarian stimulation, and the ultrasonographic assessment of follicular development and uterine receptivity.

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Abstract

During the early follicular phase in the menstrual cycle, antral follicle sizes are often markedly heterogeneous. These follicular size discrepancies may, at least in part, result from the early exposure of FSH-sensitive follicles to gradient FSH concentrations during the preceding luteal phase. In addition, they potentially affect the results of ovarian stimulation. Indeed, pre-existing follicle size discrepancies may encumber coordinated follicular growth during ovarian stimulation, thereby reducing the number of follicles that reach maturation at once. To investigate this issue, three clinical studies were conducted to test the hypothesis that luteal FSH suppression could coordinate follicular growth. First, luteal FSH concentrations were artificially lowered by administering physiological oestradiol doses and measured follicular characteristics on the subsequent day 3. Second, it was verified whether luteal oestradiol administration could promote the coordination of follicular growth during ovarian stimulation and improve its results. Third, the effects of premenstrual gonadotrophin-releasing hormone (GnRH) antagonist administration on follicular characteristics were assessed during the early follicular phase. The results showed that luteal FSH suppression by either oestradiol or GnRH antagonist administration reduces the size and improves the homogeneity of early antral follicles during the early follicular phase, an effect that persists during ovarian stimulation. Coordination of follicular development may optimize ovarian response to short GnRH agonist and antagonist protocols, and constitutes an attractive approach to improving their outcome.

Keywords: early antral follicles, follicular synchronization, FSH, GnRH antagonist, oestradiol, ovarian stimulation

Introduction

During the first days of the follicular phase in the menstrual cycle, early antral follicles are differentially sensitive to FSH (McNatty *et al.*, 1983; Fauser and Van Heusden, 1997) and exhibit dissimilar sizes, which range from 2 to 8 mm in diameter (Gougeon *et al.*, 1983). Under physiological conditions, both of these interrelated phenomena are possibly implicated in the establishment of follicular dominance. Although it is conceivable that complex intrafollicular mechanisms combine to determine follicular sensitivity to FSH (Gougeon, 1996; De Felici *et al.*, 2005), compelling evidence indicates that larger follicles are more responsive to this hormone than are smaller follicles (Hillier *et al.*, 1980; McNatty *et al.*, 1983; Fauser and Van Heusden, 1997). The mechanisms underlying the heterogeneity of antral follicle sizes during the early follicular phase remain unclear. A possible explanation for this phenomenon involves the exposure of early antral follicles to gradient FSH concentrations during the late luteal phase. During the last days of the menstrual cycle, paralleling demise of the corpus luteum, FSH concentrations increase progressively (Mais *et al.*, 1987; Roseff *et al.*, 1989) to preserve antral follicles from atresia and ensure their subsequent growth (Chun *et al.*, 1996;

Scott *et al.*, 2004). According to their inherent sensitivity to FSH, it is possible that some early antral follicles are able to respond to lower amounts of FSH than others, and therefore to start their development during the late luteal phase (Klein *et al.*, 1996). The premature, gradual exposure of follicles to FSH may accelerate the development of more sensitive follicles and accentuate size discrepancies observed during the first days of the subsequent cycle.

This physiological context is by no means without consequences on controlled ovarian stimulation outcome. In ovarian stimulation, most early antral follicles are required to grow coordinately in response to exogenous gonadotrophins to accomplish simultaneously functional and morphological maturation. Marked follicular size discrepancies at the end of ovarian stimulation may be counterproductive, since they imply that a substantial fraction of FSH-sensitive follicles fail to undergo satisfactory maturation, which reduces the number of viable oocytes and embryos and the probability of conception (Devreker *et al.*, 1999). Hence, to optimize the results of controlled ovarian stimulation, it is plausible that the physiological size heterogeneity of early antral follicles should be primarily overcome.

Indeed, during the last 15 years, a number of attempts have been made to abridge the duration and complexity of ovarian stimulation and to improve its patient-friendliness as compared with the reference long gonadotrophin-releasing hormone (GnRH) agonist protocols for IVF–embryo transfer. Among the most relevant of these ranks the development of short GnRH agonist (Hazout *et al.*, 1983; Macnamee *et al.*, 1989) and GnRH antagonist (Diedrich *et al.*, 1994; Olivennes *et al.*, 1994) regimens. Some clinical trials have subsequently raised doubts concerning the effectiveness of these simplified approaches as compared with long GnRH agonist protocols (Tan *et al.*, 1992; Cramer *et al.*, 1999; Borm *et al.*, 2000; Al-Inany *et al.*, 2001). Nevertheless, the explanation for the poorer IVF–embryo transfer outcome achieved with these alternative protocols still remain unclear. In an effort to clarify this issue, it was noticed that both approaches to ovarian stimulation share common features that singularize them as compared with long GnRH agonist protocols. These include a shorter course of multiple follicles to maturation and smaller number of oocytes and embryos obtained (Tan *et al.*, 1992; Cramer *et al.*, 1999; Borm *et al.*, 2000; Al-Inany *et al.*, 2001). Based on this clinical background, it was postulated that the differential efficacy between long and short GnRH agonist and antagonist protocols could be, at least in part, related to pre-existing differences in the characteristics of early antral follicles before the start of gonadotrophin administration (Fanchin *et al.*, 2003a,b).

To challenge the hypothesis that such size heterogeneities among antral follicles result from their early and progressive exposure to FSH during the luteal phase, it was decided to artificially lower endogenous luteal FSH secretion by administering either oestradiol (Fanchin *et al.*, 2003a,b) or GnRH antagonist (Fanchin *et al.*, 2004) and to measure follicular and hormonal profiles during the subsequent follicular phase. These hormonal pretreatments could be helpful in coordinating antral follicle growth and optimizing controlled ovarian stimulation for IVF–embryo transfer. The present article summarizes recent experience with both approaches.

Luteal oestradiol administration

In a first study (Fanchin *et al.*, 2003a), 66 female volunteers, 20–41 years of age, were prospectively investigated. All of them had regular, ovulatory menstrual cycles every 25–35 days, both ovaries present, no current or past diseases affecting ovaries or gonadotrophin or sex steroid secretion, clearance or excretion, body mass indexes (BMI) ranging from 18 to 27 kg/m^2, no current hormone therapy, and adequate visualization of ovaries in transvaginal ultrasound scans. Due to personal reasons ($n = 4$) or major protocol violation ($n = 2$), six women did not complete the two subsequent observation cycles required by the protocol and were excluded from the analysis. Therefore, the population studied was limited to 60 participants undergoing 120 study cycles.

On day 3 of their menstrual cycles (baseline/day 3), all women underwent blood sampling for serum FSH, inhibin B, and oestradiol measurements and ultrasound scans of their ovaries. Subsequently, women were randomized to receive luteal oestradiol treatment or to serve as controls. Participants who were included in the oestradiol-treated group ($n = 30$) received micronized 17β-oestradiol oral tablets (4 mg/day; Provamès, Cassenne Laboratories, Puteaux, France), in the evening at 8 p.m., from day 20 of the same cycle until day 2 of their next cycle. Participants who were included in the control group ($n = 30$) remained untreated. On day 1 of oestradiol discontinuation in oestradiol-treated women (oestradiol/day 3) or on day 3 of the subsequent cycle in control women (control/day 3), similar hormonal and ultrasonographic measurements to the preceding cycle (baseline/day 3) were performed. In addition, participants were asked to compute their baseline and subsequent menstrual cycle lengths and to report possible subjective changes in menstrual bleeding characteristics. In oestradiol-treated women, compliance of treatment was monitored to detect any protocol violation.

Ultrasound scans were performed using a 4.5–7.2 MHz multi-frequency transvaginal probe (Siemens Elegra; Siemens S.A.S., Saint-Denis, France) by one single operator, who was not aware of the treatment schedule or the hormonal results. The objective of ultrasound examinations was to evaluate the number and sizes of early antral follicles and to calculate mean ovarian volume. All follicles that measured 2–12 mm in mean diameter (mean of two orthogonal diameters) were considered. In an attempt to optimize the reliability of ovarian follicular assessment, the ultrasound scanner used was equipped with a tissue harmonic imaging system (Thomas and Rubin, 1998), which allowed improved image resolution and adequate recognition of follicular borders. Ovarian volumes, calculated according to the formula for an ellipsoid (0.526 × length × height × width) (Sharara and McClamrock, 1999), were the mean volume for both ovaries. Intra-analysis coefficients of variation (CV) for follicular and ovarian measurements were <5% and their lower limit detection 0.1 mm respectively.

All blood samples were obtained by venipuncture and serum was separated and frozen in aliquots at –20°C for subsequent centralized analysis. Serum FSH was measured by an immunometric technique using an Amerlite kit (Ortho Clinical Diagnostics, Strasbourg, France). Intra-assay and interassay

CV were respectively 5 and 7% and lower limit of detection was 0.1 mIU/ml for FSH. Serum inhibin B was determined by double antibody enzyme-linked immunosorbent assay (Serotec, Varilhes, France) as previously described (Groome *et al.*, 1996). The lower limit for detection was 10 pg/ml, and intra-assay and interassay CV were <6 and <9% for inhibin B respectively. Serum oestradiol was determined by an immunometric technique using an oestradiol-60 Amerlite kit (Ortho Clinical Diagnostics, Strasbourg, France). The lower limit for detection was 14 pg/ml, and intra-assay and interassay CV were 8 and 9% for oestradiol respectively.

Measure of central tendency used was the mean and measure of variability was standard deviation (SD). Due to the pairwise design of this study, data from each participant on oestradiol/day 3 or on control/day 3 were compared with corresponding data for the same participant on baseline/day 3 by using the paired Student's *t*-test. To evaluate the magnitude of follicular size discrepancies from baseline/day 3 to oestradiol/day 3 and from baseline/day 3 to control/day 3, the homogeneity of variances was tested using the Levene test for equal variances (Levene, 1960). This test is less sensitive than *F*-tests to departures from normality and allows the comparison of dispersion of data around the mean independently of mean values. In addition, SD/mean ratios for follicular sizes were also calculated. The present crossover study was powered to detect anticipated differences of 0.5 mm for follicular sizes and 2 mm for ovarian volume calculation at >80% power at 0.05 significance concentration. A *P*-value <0.05 was considered statistically significant.

Follicular and ovarian measurement results are summarized in **Table 1**. As expected, the number of antral follicles did not change significantly from one cycle to another in oestradiol-treated women and in controls. In contrast, a significant reduction of mean follicular sizes was observed in oestradiol-treated women, from baseline/day 3 to oestradiol/day 3 ($P < 0.001$), but not in controls. In agreement with this, mean ovarian volume decreased significantly in women treated with oestradiol and remained unchanged in controls ($P < 0.02$). In addition, a remarkable attenuation of follicular size discrepancies was observed on oestradiol/day 3 as compared with baseline/day 3 ($P < 0.001$). This phenomenon was not seen in controls, between baseline/day 3 and control/day 3. Consistently, SD/mean ratios for follicular sizes were significantly lower ($P < 0.001$) on oestradiol/day 3 than on baseline/day 3 but not on control/day 3 as compared with baseline/day 3, which confirms the improvement in follicular size homogeneity observed in oestradiol-treated women. Incidentally, it is noteworthy that ultrasonographic measurements made on baseline/day 3 were strictly similar in women included in the oestradiol-treated and control groups.

Hormonal results are also presented in **Table 1**. In women who were administered oestradiol during the luteal phase, serum inhibin B concentrations were significantly lower on oestradiol/day 3 as compared with baseline/day 3, whereas no significant longitudinal change in inhibin B concentrations was noted in controls. As expected, oestradiol administration raised serum oestradiol to concentrations comparable to those observed during the late follicular phase of the menstrual cycle (114 ± 57 pg/ml on oestradiol/day 3). Serum oestradiol concentrations did not vary significantly in controls, from baseline/day 3 to control/day 3. Administration of oestradiol lowered serum FSH concentrations on oestradiol/day 3 as compared with baseline/day 3. However, in women who did not receive oestradiol treatment, serum FSH concentrations remained steady from one cycle to the other. Coefficients of variation for FSH from the first to the second cycle were significantly higher in oestradiol-treated women than in controls (40 versus 17%, $P < 0.01$). As for ultrasonographic measurements, hormonal results obtained on baseline/day 3

Table 1. Ultrasonographic and hormonal results during two consecutive menstrual cycles in women receiving or not receiving oestradiol during the luteal phase.

	Oestradiol-treated group			*Control group*		
	Baseline day 3	*Oestradiol day 3*	P-*value*	*Baseline day 3*	*Control day 3*	P-*value*
No. follicles (range)	10.4 ± 4.3 (3–19)	10.3 ± 4.9 (2–20)	NS	10.6 ± 4.1 (3–20)	10.5 ± 3.9 (3–20)	NS
Mean follicular size (mm)	4.9 ± 1.0	3.7 ± 0.5	<0.001	4.9 ± 0.8	5.0 ± 0.8	NS
SD/mean of follicular sizes	0.40	0.23	<0.001	0.40	0.39	NS
Mean ovarian volume (cm^3)	6.1 ± 3.0	5.1 ± 2.6	<0.02	6.1 ± 2.3	6.2 ± 2.1	NS
Serum inhibin B (pg/ml)	71 ± 32	34 ± 28	<0.001	77 ± 23	75 ± 21	NS
Serum oestradiol (pg/ml)	47 ± 29	114 ± 57	<0.001	43 ± 29	38 ± 22	NS
Serum FSH (mIU/ml)	7.3 ± 3.3	4.3 ± 1.9	<0.001	6.9 ± 2.6	7.5 ± 2.7	NS

NS: not significant; SD: standard deviation.

were closely similar in women receiving oestradiol as compared with those who served as controls.

As anticipated, the oestradiol-treated and the control groups were comparable in regard to ages of women (33.3 ± 0.6 versus 33.3 ± 0.5 years) and BMI (21.8 ± 0.4 and 21.8 ± 0.3 kg/m^2). A significant lengthening of mean menstrual cycle duration was observed in participants receiving oestradiol (29.4 ± 1.3 days, $P < 000.1$) as compared with their baseline cycles (27.8 ± 1.2 days). This phenomenon was not observed in controls (28.0 ± 1.1 versus 27.9 ± 0.8 days respectively). Moreover, oestradiol treatment did not alter baseline cycle length in oestradiol-treated (27.8 ± 1.2 days) as compared with controls (27.9 ± 0.8 days). Participants did not refer any significant change on their menstrual bleeding characteristics in oestradiol-treated as compared with baseline cycles.

The second study on the effect of luteal of oestradiol administration on antral follicle growth (Fanchin *et al.*, 2003b) prospectively analysed 100 female volunteers, 25–38 years of age, who met similar inclusion criteria as the preceding study (Fanchin *et al.*, 2003a). Indications for IVF–embryo transfer were male factor (58%), tubal factor (27%), endometriosis (2%), or unexplained infertility (13%). Intracytoplasmic sperm injection was programmed in 46% of cases. Due to personal reasons ($n = 4$) or major protocol violation ($n = 6$), 10 women were excluded from the analysis. The population analysed was therefore limited to 90 participants.

Women randomly received luteal oestradiol treatment or served as controls. Those included in the luteal oestradiol group ($n = 47$) received micronized 17β-oestradiol oral tablets (4 mg/day; Provamès, Cassenne Laboratories, Puteaux, France), in the evening at 8 p.m., from day 20 of the same cycle until day 2 of their next cycle. Patients included in the control group ($n = 43$) remained untreated during the luteal phase.

On the first day of oestradiol discontinuation (cycle day 3) in oestradiol-treated group or on cycle day 3 in controls, r-FSH treatment (Gonal-F; Serono Laboratories, Saint Cloud, France) was started at a fixed dose set at 225 IU/day, SC, for 5 days. Further r-FSH administration was adjusted according to usual parameters of follicle growth determined by serum oestradiol concentrations and ultrasound monitoring. When at least one follicle exceeded 13 mm in diameter (de Jong *et al.*, 2000), a potent, third-generation GnRH antagonist, cetrorelix acetate (Cetrotide, 3 mg; Serono Laboratories), was administered subcutaneously in a single dose at 8 p.m. An intramuscular injection of 10,000 IU of human chorionic gonadotrophin (HCG, Gonadotrophine Chorionique 'Endo'; Organon Laboratories, Saint-Denis, France) was performed when at least five follicles ≥16 mm in diameter were obtained. Follicular sizes (mean of two orthogonal diameters) exceeding 20 mm in diameter were avoided as far as possible. Cycle cancellation for inadequate ovarian response to ovarian stimulation was decided when <5 follicles ≥12 mm in diameter were observed after 10 days of r-FSH treatment. Oocytes were retrieved 36 h after HCG administration by transvaginal ultrasound-guided aspiration. Adequate embryo quality was defined as embryos having uniform sized and shaped blastomeres, ooplasm having no granularity and a maximum fragmentation of 10%. All embryo transfers were performed 2 days after oocyte retrieval using a Frydman catheter (CCD Laboratories, Paris, France). Luteal phase was supported with 400 mg of micronized progesterone (Estima Gé; Effik Laboratories, Bièvres, France) administered daily (200 mg in the morning, 200 mg in the evening) by vaginal route starting on the day of embryo transfer.

Ultrasound scans were performed using a 4.5–7.2 MHz multi-frequency transvaginal probe (Siemens Elegra; Siemens S.A.S., Saint-Denis, France) in the morning at approximately 8 a.m. by operators who were not aware of the treatment schedule. In addition to usual ultrasonographic monitoring of ovarian stimulation, by design, all women underwent a detailed ultrasound scan of their ovaries on day 8 of r-FSH treatment. During this examination, the number and sizes (mean of two orthogonal diameters) of antral follicles were evaluated. Among them, follicles ≥8 mm in diameter were considered as growing. Inter- and intra-analysis CV for follicular measurements were <5% and their lower limit detection 0.1 mm respectively.

Serum hormonal (oestradiol, progesterone, and LH) measurements that were performed on baseline (just before the start of r-FSH administration), on days 6 and 8 of r-FSH therapy, and on the day of HCG were included in the present analysis. Statistical methodology was conducted as in the previous study (Fanchin *et al.*, 2003a).

The population included in the oestradiol-treated and control groups was similar with regard to women's ages (median, 33 years, range 26–38 versus 33 years, range 25–38 respectively), indications for IVF–embryo transfer (male factor, 62 versus 54%; tubal factor, 21 versus 32%; endometriosis, 2 versus 2%; unexplained infertility, 15 versus 12% respectively), duration of infertility (4.3 ± 0.2 versus 4.1 ± 0.2 years respectively), rank of the current IVF–embryo transfer attempts (2.6 ± 0.3 versus 2.1 ± 0.2 respectively), average menstrual cycle length (29.5 ± 0.4 versus 29.4 ± 0.4 days respectively), and ovarian status assessment by day 3 serum FSH (6.1 ± 0.2 versus 6.2 ± 0.2 mIU/ml respectively) and oestradiol (31.6 ± 2.4 versus 29.1 ± 2.6 pg/ml respectively) measurements performed within 3 months before inclusion in the protocol. Luteal oestradiol treatment lasted 11.3 ± 0.6 days. This treatment was well tolerated by patients, who did not experience any unwanted side effects. Luteal oestradiol administration did not alter the expected onset of menstrual bleeding.

Follicular development characteristics and embryologic data in both groups are presented in **Table 2**. As expected, the number of growing follicles was similar in the two groups on day 8. In contrast, a significant reduction was observed in the mean follicular sizes on day 8 of r-FSH treatment in the luteal oestradiol as compared with the control group ($P < 0.001$). In addition, calculation of homogeneity of variances indicated a noticeable attenuation of follicular size discrepancies in the luteal oestradiol as compared with the control group on the same observation day ($P < 0.01$). In line with this, CV of follicular sizes on day 8 were slightly, yet significantly, lower in the luteal oestradiol than in the control group ($P < 0.02$), which further confirms the attenuation in follicular size disparity after oestradiol pretreatment. No woman in both groups received GnRH antagonist before day

Table 2. Follicular development during ovarian stimulation and embryological results in women pretreated or not pretreated with oestradiol during the luteal phase.

	Luteal oestradiol group	*Control group*	P-*value*
No. follicles >10 mm on day 8	16.4 ± 1.0	16.8 ± 0.9	NS
Mean follicular size on day 8 (mm)	9.9 ± 0.2	11.1 ± 0.3	<0.001
CV of follicular sizes on day 8	0.22	0.26	<0.02
Day of GnRH antagonist administration	9.1 ± 0.2	8.5 ± 0.2	<0.01
Day of HCG administration	11.9 ± 0.2	10.8 ± 0.2	<0.001
No. follicles ≥16 mm on day of HCG	9.9 ± 0.5	7.9 ± 0.5	<0.01
No. mature oocytes	9.3 ± 0.7	7.3 ± 0.5	<0.03
No. available embryos	6.4 ± 0.6	4.6 ± 0.3	<0.01
No. embryos transferred	2.6 ± 0.1	2.7 ± 0.1	NS
Clinical pregnancy rates/cycle (%)	34	25	NS

NS: not significant; CV: coefficient of variation.

8. However, both cetrorelix acetate and HCG were administered later in the luteal oestradiol group as compared with the control group. Luteal oestradiol patients tended to require a higher r-FSH dose than controls, but not significantly (2674 ± 91 versus 2463 ± 100 IU respectively). Six ovarian stimulation cycles (three in each group) had to be cancelled due to unexpected, inadequate response to ovarian stimulation. On the day of HCG administration, more follicles had exceeded 15 mm in diameter in the oestradiol-treated as compared with the control group. Consistently, luteal oestradiol pretreatment was associated with more mature oocytes and available embryos than in the control group. Intracytoplasmic sperm injection was performed in 43 and 50% of cases included in luteal oestradiol and control groups respectively. Whereas the number of embryos transferred was similar in both groups, a higher prevalence of good quality embryos transferred was observed in women pretreated with oestradiol as compared with controls (63 versus 44% respectively, $P < 0.03$), which probably reflects the improved embryo selection from a larger embryo cohort. Although the present investigation was not powered to detect differences in IVF–embryo transfer outcome, a trend for increased pregnancy rates in luteal oestradiol group was incidentally observed.

As an expected result of luteal oestradiol administration, serum oestradiol concentrations remained higher in the oestradiol-treated as compared with the control group (139 ± 54 versus 34 ± 8 pg/ml) at baseline. Conversely, on day 6 and on day 8, serum oestradiol reached slightly, but significantly ($P < 0.05$), lower concentrations in patients pretreated with luteal oestradiol, probably reflecting a slower pace of follicle development. On the day of HCG, however, serum oestradiol concentrations in the luteal oestradiol group tended to exceed control values, which is in keeping with the larger number of mature follicles obtained in that group. Serum progesterone and LH concentrations remained similar in both groups throughout r-FSH treatment but, as for oestradiol, progesterone showed a trend to higher concentrations in the luteal oestradiol group as compared with the control group.

Premenstrual GnRH antagonist administration

In the third study (Fanchin *et al.*, 2004), another approach was tested that aimed at sparing early antral follicles from the putative uncoordinating effects of luteal FSH. It involved administrating a potent GnRH antagonist, cetrorelix acetate, during the late luteal phase. At a 3-mg dose, this molecule has been shown to bring down endogenous FSH secretion for at least 2 days (Erb *et al.*, 2001), which opportunely corresponds to the timing during which luteal FSH reaches significant serum concentrations (Mais *et al.*, 1987; Roseff *et al.*, 1989). For this, follicular and hormonal profiles were assessed on day 2 in two consecutive menstrual cycles pretreated or not by GnRH antagonist during the late luteal phase.

Thirty female volunteers, 20–41 years of age, were prospectively studied. All participants had similar inclusion criteria to the preceding studies. An informed consent was obtained from all women and this investigation received the approval of the internal Institutional Review Board. Due to major protocol violation ($n = 4$) or personal reasons ($n = 1$), five women did not go through the two subsequent observation cycles required by the protocol and had to be excluded from the analysis. Therefore, the population studied was limited to 25 participants undergoing 50 study cycles.

On day 2 of their menstrual cycles (control/day 2), women underwent blood sampling for serum FSH, inhibin B, and oestradiol measurements at approximately 9 a.m. Later in the morning, ultrasound scans of their ovaries were performed. On day 25, participants received a single injection of GnRH antagonist (cetrorelix acetate, 3 mg; Cetrotide, Serono Laboratories, Boulogne, France), in the evening at approximately 8 p.m. On day 2 in the subsequent cycle (premenstrual GnRH antagonist/day 2), similar hormonal and ultrasonographic measurements as in the preceding cycle (control/day 2) were performed. It was decided to perform

follicular hormonal measurements on day 2 of the cycle because, in controlled ovarian stimulation protocols using GnRH antagonists, exogenous gonadotrophin administration usually starts on day 2. Furthermore, participants were asked to compute their control and subsequent menstrual cycle lengths and to report possible subjective changes in menstrual bleeding characteristics.

Ultrasonographic measurements in the present study followed similar methodological characteristics to those reported elsewhere (Fanchin *et al.*, 2003a,b). The objective of ultrasound examinations was to evaluate the number and sizes of early antral follicles. All follicles that measured 2–12 mm in mean diameter (mean of two orthogonal diameters) were considered. Serum FSH, inhibin B, and oestradiol concentrations were determined and data were statistically analysed according to similar methodology as described previously (Fanchin *et al.*, 2003a,b).

Mean ages of the women were 33.0 ± 4.5 years and mean BMI values were 21.5 ± 3.0 kg/m^2. Baseline menstrual cycle duration was 28.1 ± 0.6 days, which corresponded to a mean time elapsed from GnRH antagonist administration until the onset of menstrual bleeding of 3.1 ± 0.6 days (range, 2–4 days). Participants did not report any remarkable change in their menstruation characteristics, or in the onset of menstrual bleeding, in GnRH-antagonist-pretreated cycles as compared with control cycles. However, a slight yet significant lengthening of GnRH-antagonist-pretreated cycles as compared with the baseline cycle (28.8 ± 1.0 versus 28.1 ± 0.6 days, $P < 0.004$) was observed.

Follicular measurement results are summarized in **Table 3.** Mean time elapsed between GnRH antagonist administration and ultrasonographic assessment of early antral follicles was 4.1 ± 0.6 days (range, 3–5 days). A significant reduction was observed in the mean follicular sizes in GnRH-antagonist-pretreated cycles as compared with baseline cycles ($P < 0.001$). In addition, both the calculation of homogeneity of variances and CV of follicular size indicated a noticeable attenuation of follicular size discrepancies on premenstrual GnRH antagonist/day 2 ($P < 0.001$). Further, no significant differences in the magnitude of follicular size modifications were identified, irrespective of the time elapsed between GnRH antagonist and ovarian measurements. Hormonal results are also presented in **Table 3.** As expected, cetrorelix acetate significantly decreased serum FSH concentrations on premenstrual GnRH antagonist/day 2 ($P < 0.001$). In line with this, and consistent with the significant reduction in follicular sizes, serum oestradiol and inhibin B concentrations were significantly lower on premenstrual GnRH antagonist/day 2 than on control/day 2 ($P < 0.001$ and $P < 0.01$ respectively).

Discussion

The present article summarizes three studies aimed at challenging the hypothesis that developmental asynchrony of early antral follicles is possibly due to the gradual FSH elevation occurring during the late luteal phase. Progressive FSH elevation may promote asynchronous growth of follicles because of their dissimilar intrinsic sensitivity to FSH (McNatty *et al.*, 1983; Fauser *et al.*, 1997). The first two trials showed that luteal oestradiol administration, through its putative suppressive effect on FSH secretion (le Nestour *et al.*, 1993; de Ziegler *et al.*, 1998), attenuates antral follicle size heterogeneity. In the third investigation (Fanchin *et al.*, 2004), it was shown that a single administration of cetrorelix acetate, 3 mg, during the last days of the luteal phase in regularly menstruating volunteers effectively reduces both the size discrepancies and the mean diameter of early antral follicles during the subsequent follicular phase. The observed lengthening of the ensuing menstrual cycle after either luteal oestradiol (Fanchin *et al.*, 2003a) or premenstrual GnRH antagonist (Fanchin *et al.*, 2004) administration is in keeping with this effect, and presumably results from a longer growth course of smaller antral follicles to ovulation, which corroborates data by other investigators (Skarin *et al.*, 1982; Mais *et al.*, 1986; Hall *et al.*, 1991).

When administered before controlled ovarian stimulation, luteal oestradiol administration effectively reduces the pace of multi-follicular growth in response to r-FSH (Fanchin *et al.*, 2003b). Indeed, a slower increase in serum oestradiol concentrations was observed during ovarian stimulation and reduced antral follicle sizes on day 8 in oestradiol-pretreated ovarian stimulation. In addition, follicles took longer to achieve maturation and required later GnRH antagonist and HCG administration in oestradiol-pretreated patients. These

Table 3. Ultrasonographic and hormonal data on day 2 during two consecutive menstrual cycles pretreated or not pretreated with gonadotrophin-releasing hormone (GnRH) antagonist during the late luteal phase.

	Control/day 2	*Premenstrual GnRH antagonist/day 2*	P-*value*
No. follicles (range)	9.4 ± 2.9 (3–19)	9.3 ± 2.2 (2–20)	NS
Mean follicular size (mm)	5.5 ± 1.0	4.1 ± 0.9	<0.001
CV of follicular sizes (%)	38	20	<0.001
Serum inhibin B (pg/m)	76 ± 33	52 ± 30	<0.01
Serum oestradiol (pg/ml)	46 ± 26	23 ± 13	<0.001
Serum FSH (mIU/ml)	6.7 ± 2.4	4.5 ± 1.9	<0.001

NS: not significant; CV: coefficient of variation.

effects may be due to an overall reduction in early antral follicle sizes at the start of r-FSH treatment. Luteal oestradiol pretreatment also fostered follicular growth coordination during controlled ovarian stimulation, as corroborated by the attenuation of follicle size discrepancies on day 8 and the increased number of follicles reaching maturation at once. These follicular effects significantly increased the number of viable oocytes and available embryos ($P < 0.03$ and $P < 0.01$ respectively). Indeed, the number of embryos produced has been shown to influence positively IVF–embryo transfer outcome (Devreker *et al.*, 1999), possibly through the optimization of embryo selection for embryo transfer. The observation that oestradiol-pretreated patients had more top-quality embryos transferred and showed a trend for improved pregnancy rates is consistent with this hypothesis.

The present data also provide alternative insights into the reported improvement of ovarian stimulation outcome with combined oral contraceptive pretreatment (Gonen *et al.*, 1990; Schoolcraft *et al.*, 1997). Indeed, due to their potent anti-FSH action, oral contraceptives may exert similar, or even stronger, coordinating effects on early follicular development as compared with luteal oestradiol or premenstrual GnRH antagonist administration. In line with this, Gonen *et al.* (1990) reported an increase in the number of mature follicles and oocytes in clomiphene citrate cycles preceded by oral contraceptives as compared with controls. Nevertheless, combined oral contraceptives have putative shortcomings, such as lengthy treatment course, menstrual bleeding postponement, and adverse effects that are not shared by the 10-day administration of physiological 17β-oestradiol doses used in the first two studies described in this paper (Fanchin *et al.*, 2003a,b). Comparative studies between the effects of oral contraceptive and luteal oestradiol or GnRH antagonist pretreatments on follicular cohort characteristics and ovarian stimulation outcome are needed to clarify this issue.

The reduction in the pace of follicular development observed after premenstrual GnRH antagonist administration is likely to result from its FSH-suppressive effect. Indeed, cetrorelix acetate has been shown to induce a rapid, transient, and dose-dependent decrease in endogenous gonadotrophins that is faster and more pronounced for LH than FSH (Erb *et al.*, 2001). Both the shorter half-life of LH (Erb *et al.*, 2001) and the possible GnRH-independent regulation of FSH secretion (Hall *et al.*, 1990, 1991) may combine to explain these differential effects on gonadotrophins. The deceleration in early follicular growth induced by cetrorelix acetate is in agreement with the reported arrest of antral follicle growth resulting from the action of another GnRH antagonist, Nal-Glu, administered during the mid-follicular phase in the menstrual cycle (Kettel *et al.*, 1991). This suggests that a noticeable impairment of follicular development may occur even in presence of incomplete endogenous FSH suppression. Consistently, in the study by Kettel *et al.* (1991), 3-day Nal-Glu antagonist administration reduced mean immunoreactive FSH concentrations by approximately 45%, whereas 3-mg cetrorelix acetate administration reportedly reduces FSH concentrations by 42% (Fanchin *et al.*, 2004). Therefore, the significant modification in early antral follicle development observed after luteal cetrorelix acetate administration leads to the inference that such treatment keeps transitorily serum FSH concentrations beneath the FSH threshold for early follicular development.

Another pertinent yet controversial (Roseff *et al.*, 1989; Hall *et al.*, 1991) issue is the possible luteolytic action of GnRH antagonists. Corpus luteum sensitivity to LH suppression presumably shows interindividual variations (Roseff *et al.*, 1989), and its degree of LH dependence has been the matter of debate (Roseff *et al.*, 1989; Hall *et al.*, 1991). As cetrorelix acetate was administered during the premenstrual phase in the present study, the occurrence of spontaneous or induced luteolysis remained indiscernible to the participants, who did not present any clinical evidence of menstrual advancement. Yet, from a practical standpoint, any acceleration in the onset of menses would not hamper the primary clinical application of the present approach, since exogenous gonadotrophin treatment for controlled ovarian stimulation can be started as early as complete corpus luteum demise occurs.

In conclusion, the possibility of reducing size differences among FSH-sensitive follicles during the early follicular phase may foster follicular growth coordination in response to exogenous gonadotrophin administration. This issue is particularly key with regard to ovarian stimulation protocols deprived of luteal FSH control, such as short GnRH agonist (Macnamee *et al.*, 1989; Hazout *et al.*, 1993) and GnRH antagonist (Diedrich *et al.*, 1994; Olivennes *et al.*, 1994) regimens. These approaches represent potential alternatives to GnRH agonist or oral contraceptive pretreatment to synchronize multi-follicular development and improve ovarian stimulation results. However, larger studies are needed to confirm whether follicular growth coordination induced by luteal oestradiol (Fanchin *et al.*, 2003a,b) and premenstrual GnRH antagonist (Fanchin *et al.*, 2004) administration improves IVF–embryo transfer pregnancy rates with GnRH antagonist or short GnRH agonist protocols.

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The Merck Manual of Diagnosis and Therapy
Section 18. Gynecology And Obstetrics
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Topics
[General]

[General]

Normal reproductive function depends on complex hormonal communication between endocrine and target organs. Normal function is essential to sexual development at puberty and to the cyclic processes of ovulation and menstruation.

The hypothalamus secretes a small peptide, gonadotropin-releasing hormone (GnRH), also known as luteinizing hormone-releasing hormone, which regulates release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland (see Fig. 234-1 and Chs. 6 and 7). LH and FSH promote maturation of ova and stimulate secretion of estrogen and progesterone from the ovaries.

Estrogen and progesterone are polycyclic compounds (with carbon atoms arranged in four rings) derived from cholesterol. They circulate in the bloodstream almost entirely bound to plasma proteins. Only unbound estrogen and progesterone appear to be biologically active. They stimulate the target organs of the reproductive system (ie, breasts, uterus, and vagina) and exert negative and positive feedback effects on the CNS-hypothalamic-pituitary unit, inhibiting and stimulating gonadotropin secretion.

Virtually all hormones are released in short bursts or pulses at intervals of 1 to 3 h, so descriptions of hormonal patterns are idealized representations. This factor must be considered when interpreting single hormonal values.

Hormone Secretion: Infancy Through Puberty

LH and FSH are elevated at birth but fall to low levels within a few months and remain low throughout the prepubertal years, with FSH generally slightly higher than LH (see Fig. 234-2).

The adrenal androgens dehydroepiandrosterone (DHEA) and DHEA sulfate begin to increase several years before puberty. These increases may be important in initiating pubic and axillary hair growth (ie, adrenarche) and other pubertal events. Because ACTH and cortisol do not increase at this time, an unidentified pituitary peptide may initiate adrenal androgen secretion.

The mechanisms initiating puberty are unclear. Central influences may inhibit the pulsatile release of GnRH during childhood, then initiate its release to induce puberty early in adolescence.

Early in puberty, a decreased sensitivity of the hypothalamus to sex hormones results in increased secretion of LH and FSH, which stimulate production of sex hormones (primarily estrogen) and development of secondary sexual characteristics. Secretion of LH and FSH increases only during sleep at first, then throughout the 24-h period. Patterns of increased basal LH and FSH levels are different for boys and girls, but in both, LH increases more than FSH.

Puberty

The sequence of events by which a child reaches sexual maturity and growth.

Physical changes of puberty occur sequentially during adolescence. Breast budding in girls is usually the first noticeable change, followed closely by the appearance of pubic and axillary hair (see Fig. 234-3). Menarche (a female's first menstrual period) occurs about 2 yr after breast budding. The pubertal growth spurt typically begins before breast budding but is seldom recognized. Girls reach peak height velocity early in puberty, before menarche; growth potential is limited after menarche. Habitus changes, and the percentage of body fat increases.

The age at which puberty begins varies, apparently influenced by general health, nutrition, socioeconomic conditions, and genetic factors. In industrialized nations, the age of onset has decreased consistently; eg, in Western Europe, the age at menarche decreased by 4 mo for each decade between 1850 and 1950 but has not decreased in the last 4 decades. Moderate obesity is associated with earlier menarche, and menarche is commonly delayed in severely underweight and malnourished girls. Such observations suggest that a critical body weight is necessary for menarche. Puberty also occurs early among girls living in urban areas, blind girls, and girls whose mothers matured early.

Ovarian Follicular Development

By the 6th wk of fetal development, primordial germ cells (oogonia) have migrated by ameboid movement from their site of origin in the yolk sac to the genital ridges (presumptive ovaries). Oogonia proliferate markedly by mitosis through the 4th mo, after which most undergo atresia. During the 3rd mo, some cells begin to divide by meiosis rather than mitosis, and by the 7th mo, all viable cells are arrested in the diplotene stage of meiotic prophase; these cells are primary oocytes. Between 7 and 9 mo, the fetal ovary is organized, and each oocyte becomes a part of a primordial follicle, which consists of a basement membrane, a single layer of squamous epithelial granulosa cells, and one oocyte. Primordial follicles constitute the resting follicle pool and are either initiated into a growing pool (from which all mature follicles develop) or undergo atresia. The mechanisms that initiate follicular and oocyte growth are unclear but do not require gonadotropins.

The human female is born with a limited number of ova, 99.9% of which will be eliminated by atresia. Because each oocyte remains arrested in meiotic prophase until it is ovulated, it is one of the longest-lived cells in the body (from embryo to about 50 yr). The long life span may account for the increased incidence of genetically abnormal pregnancies among older mothers.

During a woman's reproductive years, several follicles in the growing pool are recruited each cycle, and only one is usually selected for ovulation (see Fig. 234-4). It develops into a graafian (preovulatory) follicle, which can respond to the midcycle LH surge. The mechanism of selection is unknown.

The graafian follicle contains an antrum (fluid-filled cavity), created by proliferating granulosa cells, which secrete fluid and mucopolysaccharides. The increase in the follicle's size is due primarily to accumulation of follicular fluid under the control of FSH, which also induces the development of specific LH receptors on granulosa cells. LH receptors are responsible for the stimulation of progesterone secretion before ovulation and for continued production of progesterone in the luteal phase. The granulosa cells in the follicle also develop specific membrane receptors for prolactin, which decrease in number as the follicle matures; their physiologic role is unclear.

Menstrual Cycle

Menstruation is the cyclic, approximately monthly vaginal discharging of sloughed endometrium that occurs throughout a woman's reproductive life; the bloody discharge is called menses or menstrual flow.

The first day of menses is day 1 of a menstrual cycle. The average duration of menses is 5 (± 2) days. The median menstrual cycle length is 28 days, but only 10 to 15% of cycles are exactly 28 days long; the normal range for an ovulatory cycle is about 25 to 36 days. Generally, variation is maximal and intermenstrual intervals are longest in the years immediately after menarche and before menopause, when anovulatory cycles are more common. Blood loss per cycle averages 130 mL (range, 13 to 300 mL) and is usually greatest on the 2nd day. A saturated pad or tampon absorbs 20 to 30 mL. Menstrual blood does not usually clot (unless bleeding is very heavy), probably because of fibrinolysin and other factors inhibiting clotting.

The menstrual cycle can be divided into three phases on the basis of endocrine events (see Fig. 234-5). The **follicular (preovulatory) phase** extends from the first day of menses to the day before the preovulatory LH surge; its length is the most variable of the phases. During the first half of this phase, FSH secretion is increased slightly, stimulating growth of a cohort of 3 to 30 follicles that have been recruited for accelerated growth during the last days of the preceding cycle. As FSH levels decline, one of the recruited follicles is selected for ovulation; it matures, and the others undergo atresia. Circulating LH levels rise slowly, beginning 1 to 2 days after the increase in FSH. Estrogen and progesterone secretion by the ovaries is relatively constant and remains low early in this phase.

About 7 to 8 days before the LH surge, ovarian secretion of estrogen, particularly of estradiol, by the selected follicle increases slowly at first, then accelerates, generally peaking on the day before the LH surge. The rise in estrogen is accompanied by a slow but steady increase in LH and a decrease in FSH levels. LH and FSH levels may diverge because FSH secretion is preferentially inhibited by estrogens (compared with LH secretion) and is specifically inhibited by inhibin. Just before the LH surge, progesterone levels also begin to increase significantly.

In **the ovulatory phase,** a series of complex endocrine events culminates in the LH surge--the massive preovulatory release of LH by the pituitary gland. The LH surge results in part from positive estrogen feedback. A smaller increase in FSH secretion occurs simultaneously, but its significance is not understood. As LH levels increase, estradiol levels decrease, but progesterone levels continue to increase. The LH surge typically lasts 36 to 48 h and consists of multiple large bursts of LH released in pulses. The LH surge, which results in complete maturation of the follicle, is necessary for ovulation--release of the ovum from the mature graafian follicle--which usually occurs 16 to 32 h after onset of the surge. The mechanism of ovulation is unclear.

During the LH surge, the follicle swells and bulges above the ovarian epithelium. A stigma, or avascular spot, appears on the follicle's surface. A small vesicle forms on the stigma, then breaks, and the cumulus mass (the oocyte and some surrounding granulosa cells) is extruded. Prostaglandin production by the follicle, perhaps regulated by LH and/or FSH, appears essential for ovulation. Proteolytic enzymes in granulosa cells and in epithelial cells overlying the preovulatory follicle, local growth factors, and cytokines may be important. The oocyte remains arrested in meiotic prophase until after the LH surge. Within 36 h of the LH surge, the oocyte completes the first meiotic division, when each cell receives 23 chromosomes of the original 46 and the first polar body is extruded. The 2nd meiotic division, when each chromosome divides longitudinally with identical pairs, is not completed and the 2nd polar body is not extruded unless the egg is penetrated by a spermatozoon.

In **the luteal (postovulatory) phase,** the granulosa and theca cells, which make up the follicle, reorganize to form the **corpus luteum** (yellow body), for which the phase is named. The length of this phase is the most constant, averaging 14 days in nonpregnant women and ending with day 1 of menses. The length corresponds to the functional life span of the corpus luteum, which secretes progesterone and estradiol for about 14 days, then degenerates unless pregnancy ensues. The corpus luteum supports the implantation of the fertilized ovum by secreting progesterone in increasing quantities, peaking at about 25 mg/day 6 to 8 days after the LH surge. Because progesterone is thermogenic, basal body temperature increases by 0.5° C (0.9° F) during the luteal phase and remains elevated until menstruation. Regulation of the life span of the corpus luteum is poorly understood, but prostaglandins and insulin-like growth factor II may be involved.

If fertilization occurs, human chorionic gonadotropin (hCG) from the fertilized ovum supports the corpus luteum until the fetoplacental unit can support itself endocrinologically. hCG is structurally and functionally similar to LH; however, pregnancy tests typically use antibodies that are specific to the subunit of hCG and that have little if any cross-reactivity with LH.

During most of the luteal phase, circulating LH and FSH levels decrease and are low, but they begin to increase with menstruation (next cycle).

Cyclic Changes in Other Reproductive Organs

Endometrium: Cyclic changes in the endometrium culminate in menstrual bleeding. The endometrium, which consists of glands and stroma, has three layers: the basal layer, the intermediate spongiosa layer, and the superficial layer of compact epithelial cells that line the uterine cavity. The basal layer is not sloughed during menses and regenerates the other two layers, which are sloughed. Histologic changes during the menstrual cycle are characteristic, and endometrial biopsies may be used to accurately determine the stage of the cycle and assess tissue response to gonadal steroids.

Early in the follicular phase, the endometrium is thin (about 2 mm), with narrow, straight glands lined with low columnar epithelium. The stroma is dense. As estradiol levels increase late in the follicular phase, the endometrium grows rapidly and progressively with extensive mitoses (ie, regeneration from the basal layer) to 11 mm, the mucosa thickens, and the tubular glands lengthen more, becoming coiled. The endometrium can be seen using transvaginal ultrasonography; it characteristically has a trilaminar pattern in this phase, but after ovulation, it is homogeneous.

During the luteal phase, the tubular glands, under the influence of progesterone, dilate, fill with glycogen, and become secretory, and stromal vascularity increases. As estradiol and progesterone levels decrease late in the luteal phase, the stroma becomes edematous, necrosis of the endometrium and its blood vessels occurs, and endometrial bleeding ensues.

Cervix: Vascularity, congestion, edema, and mucus secretion increase progressively during the follicular phase. The external os opens to 3 mm at ovulation, then decreases to 1 mm. Increasing levels of estrogen lead to a 10- to 30-fold increase in the quantity of cervical mucus. The characteristics of cervical mucus are clinically useful in evaluating the stage of the cycle and hormonal status of a patient. Mucus elasticity (spinnbarkeit) increases, as does ferning (palm leaf arborization of mucus dried on a glass slide and examined microscopically), which becomes prominent just before ovulation. Ferning indicates increased NaCl in cervical mucus, an effect of estrogen. During the luteal phase, progesterone causes the cervical mucus to thicken, become less watery, and lose its elasticity and ability to fern.

Vagina: Proliferation and maturation of the vaginal epithelium are influenced by estrogen and progesterone. When ovarian estrogen secretion is low early in the follicular phase, the vaginal epithelium is thin and pale. As estrogen levels increase during the follicular phase, squamous cells mature and become cornified so that the epithelium thickens. During the luteal phase, the number of precornified intermediate cells increases, and the number of leukocytes and amount of debris increase as mature squamous cells are shed. Changes in the vaginal epithelium can be quantitated histologically and can be used as a qualitative index of estrogenic stimulation.

Neuroendocrine Regulation of the Menstrual Cycle

The pulsatile secretion of LH and FSH is determined by the pulsatile secretion of GnRH. The frequency and amplitude of the LH and FSH pulses are modulated by ovarian hormones and vary throughout the menstrual cycle. No separate releasing hormone for FSH has been identified. Evidence suggests that the same cells sometimes contain LH and FSH, so differential release of LH and FSH must result from interactions of various factors (eg, GnRH, estradiol, inhibin). Also, the disparate half-lives of LH (20 to 30 min) and FSH (2 to 3 h) affect circulating levels.

Of ovarian hormones, estradiol-17 is the most potent inhibitor of gonadotropin secretion, acting on the hypothalamus and the pituitary gland. Inhibin, a peptide hormone produced by ovarian granulosa cells, specifically suppresses FSH release. Removal of the ovaries results in a rapid increase in circulating LH and FSH levels; administration of estradiol to hypoestrogenic women results in a prompt decrease in these levels. However, for ovulation to occur, estradiol must exert a positive effect on gonadotropin secretion. The feedback effects of estradiol appear to be time- and dose-dependent. Early in the follicular phase, the gonadotrophs in the anterior pituitary have relatively small amounts of LH and FSH available for release from the anterior pituitary gland. Levels of estradiol (produced by the selected follicle) increase, stimulating LH and FSH synthesis but inhibiting their secretion. At midcycle, high estradiol levels exert a positive feedback effect; they, with GnRH and low but increasing quantities of circulating progesterone, induce the LH surge. Whether pulsatile release of GnRH is increased at midcycle is unknown; the midcycle surge could result from a rapid increase in the number of GnRH receptors (stimulated by estrogen) on the pituitary gonadotrophs.

Menopause, when cyclic ovarian function manifested by menstruation ceases, is discussed in Ch. 236.

Current Practices and Controversies in Assisted Reproduction

Report of a meeting on
"Medical, Ethical and Social Aspects of Assisted Reproduction"
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JEAN-NOEL HUGUES

Introduction

Within the past decade, a better understanding of the hormonal control of human folliculogenesis and a simultaneous development of DNA technologies providing new products with high purity and good clinical efficacy have allowed most physicians to use ovarian stimulation in every clinical situation where the main goal is to achieve pregnancy. Indeed, ovarian stimulation has been advocated as a common practice not only in the treatment of infertile couples with amenorrhoea or anovulation but also for couples whose infertility was related to either tubal, male or unexplained factors. For these reasons, controlled ovarian hyperstimulation (COH) has increasingly become a new tool in many situations.

The concept of controlled ovarian hyperstimulation

The concept of COH emerged from the practice of *in vitro* fertilization (IVF). Although Louise Brown was born following *in vitro* fertilization-embryo transfer (IVF-ET) in a natural cycle, it soon became clear that the pregnancy rate was greatly improved if more than one embryo was replaced in the uterus *(1,2)*. Thus, the aim of any regimen for controlled ovarian stimulation was to obtain as many follicles as possible from which good quality eggs could be collected. However, the simultaneous risks of ovarian hyperstimulation syndrome (OHSS) and multiple pregnancies have led to the adoption of a compromise between pregnancy rates and multiple follicular development, and restriction in the number of embryos transferred *(3)*. However, such a policy is not fully applicable to intrauterine insemination (IUI) and major concerns remain about the risk of multiple pregnancies when ovarian stimulation is performed in a context of *in vivo* fertilization. This critical issue will be discussed throughout this review.

Controlled ovarian stimulation protocols

Clomiphene citrate

The antiestrogenic properties of clomiphene citrate have been used and have proved to be very effective for the induction of follicular development in anovulation since 1962. However, the clinical benefit of this compound is confined to WHO Group II anovulation patients with endogenous ovarian steroidogenesis. In this situation, the ovulation and pregnancy rates achieved with this compound have justified its use as a first-line therapy in this type of anovulation. Nevertheless, some clinical and biological features may be relevant to predict the effectiveness of

clomiphene citrate to achieve ovulation or pregnancy *(4,5)*.

As administration of clomiphene citrate alone may induce a limited increase in the number of preovulatory follicles, it may be worthwhile for the management of IUI in normally ovulating patients. However, its antioestrogenic action could, theoretically, interfere with endometrial receptivity and with implantation *(6)* and, as discussed below, the rationale to use clomiphene citrate in this indication is controversial.

The use of clomiphene citrate in combination with gonadotrophins was first recommended for patients undergoing IVF. However, the effectiveness of such a regimen has been hampered by the risk of a premature spontaneous luteinizing hormone (LH) surge which occurs in about 20% of stimulated cycles and leads to IVF cancellation or impaired oocyte quality *(7)*. Therefore, gonadotrophin releasing hormone (GnRH) agonists, by preventing an untimely LH surge, have offered an effective alternative to this regimen and this approach has been used since the mid-1980s. The recent marketing authorization of GnRH antagonists, which induce an immediate suppression of endogenous LH secretion, has provided the opportunity of reconsidering the role of clomiphene citrate therapy in ovarian stimulation. The clinical effectiveness of this new regimen is now being tested.

GnRH analogues

A GnRH analogue is a peptide in which the primary structure of GnRH has been altered by the deletion or the substitution of one or more amino acids. A large number of structural analogues of GnRH have been synthesized, including both agonists and antagonists.

GnRH agonists (GnRH-a)

While GnRH agonists display a high *in vitro* biopotency, results from experimental and clinical studies have shown that repeated daily *in vivo* administration of these compounds induces a biphasic pattern of gonadotrophin secretion. Indeed, the first GnRH-a injections induce a sharp release of both gonadotrophins (the so-called "flare-up effect") followed by a state of pituitary desensitization related to both downregulation of the GnRH receptor and intracellular uncoupling. This leads to a progressive reduction in gonadotrophin synthesis which is maintained during GnRH-a administration. Both the intensity and duration of the pituitary desensitization are dose-dependent, at least for LH *(8,9)*. However, it is well recognized that, during this period of desensitization, LH radioimmunoassays do not actually reflect hormonal bioactivity. After cessation of GnRH-a administration, a refractory period to endogenous GnRH is observed, the duration of which is dependent on both the dose and the formulation of the GnRH-a.

These properties of GnRH-a have been applied in clinical practice with two main regimens of administration.

The short-term GnRH-a protocol

This regimen takes advantage of the initial rise (flare-up) of serum gonadotrophins on follicular recruitment and of the subsequent pituitary desensitization induced by daily agonist administration. Gonadotrophin administration is started in the early follicular phase. Its clinical efficacy has been previously established *(10–13)*. Several adjustments to this protocol have been suggested:

- a shorter period of GnRH-a administration, for 3 days (ultra-short protocol) *(14)* or for 7 days *(15)*, on the assumption that suppression of the endogenous LH surge may be obtained through a very short course of GnRH-a administration;
- pretreatment with progestogens during the luteal phase of the cycle preceding IVF in order to programme the patient's cycle and the timing of oocyte retrieval *(16,17)*.

The long-term GnRH-a protocol

With this regimen, both pituitary and ovarian desensitization are induced by GnRH-a administration in the early follicular or mid-luteal phase of the cycle preceding the planned IVF. Once desensitization is obtained, ovarian stimulation with gonadotrophins is started and GnRH-a injection is continued until human chorionic gonadotrophin (hCG) is administered.

GnRH-a administration is now used routinely for all patients undergoing IVF-type procedures although the early studies advocating the superiority of GnRH-a over conventional stimulation regimens were performed in patients who were poor or abnormal responders *(18)*. A meta-analysis of randomized studies has shown that GnRH-a reduces IVF cancellation rates, increases the number of oocytes recovered

and improves the clinical pregnancy rates per cycle and per embryo transfer *(19)*.

In clinical practice, the long-term protocol is the most traditional and widely used regimen, probably because it is more convenient for programming IVF. However, controversial conclusions have been drawn from studies comparing the respective effectiveness of long-term and short-term GnRH-a protocols. Some authors have reported a better outcome for patients treated with the long-term GnRH-a regimen *(20–22)*, advocating that the initial flare-up effect of the GnRH-a causes a rise in serum LH, androgens and progesterone (P) levels that might be deleterious for oocyte quality. In contrast, others have published good clinical results with the flare-up regimen *(23,24)*. Discrepancies between these results may be related to several biases in patient selection rather than to the regimen itself, since no significant difference was found in a meta-analysis of seven trials comparing both regimens *(19)*. It must be pointed out that the short-term GnRH-a regimen may provide some economic advantages in terms of reducing both agonist and gonadotrophin doses because the long protocol requires a more prolonged stimulation and administration of higher doses of exogenous gonadotrophins than the short one.

Whatever regimen is used, the long-term protocol with GnRH-a for IVF cycles does not exclude some minor concerns in clinical practice. Within the past decade, several issues have been addressed with regard to this protocol.

One of these issues concerns pituitary desensitization. The main parameters of desensitization (rapidity, intensity and duration) are critically dependent on numerous factors in the GnRH-a protocol including: which analogue is used; the time of its first administration in the cycle; the dose and duration of administration; and the formulation (see review in 25). As far as the most appropriate time of GnRH-a administration is concerned, downregulation seems to be achieved more rapidly when the GnRH-a is started in the midluteal phase. A combination of norethisterone acetate or a combination of oral contraceptive with GnRH-a has proved to be effective in preventing ovarian cyst formation *(26,27)*. Although it has been suggested that ovarian response to gonadotrophins could be reduced in patients whose pituitary downregulation is delayed *(28)*, the outcome in terms of pregnancy rates is still not clear *(29–31)*.

Another issue is related to the duration of the desensitization phase prior to ovarian stimulation. Using leuprolide acetate for 15 days prior to ovulation induction, Scott *et al. (32)* did not observe any impact of the duration of the hypoestrogenic state on the ovarian reponsiveness to gonadotrophins and the success of IVF. According to these authors, apart from the increased cost, there is no reason to believe that a patient should be stimulated as soon as pituitary desensitization and ovarian quiescence are achieved. This observation allows greater flexibility in scheduling ovulation induction cycles but seems to contradict other evidence in the literature which indicates that the implantation rate is higher in amenorrhoeic patients *(33)* as discussed below.

The slight increase in serum P levels observed at the time of hCG administration in up to 20% of stimulated cycles has caused some concern. A critical threshold P serum level of 3.1 nmol/ml on the day of hCG administration has been proposed, above which the pregnancy rate may be adversely affected (*25*). This subtle premature rise in serum P is presumed to impair endometrial receptivity rather than oocyte quality *(34–37)* and a recommendation has been made for the cryopreservation of embryos for subsequent transfer in these situations *(38)*. However, several other studies have not found a relationship between late follicular P levels and IVF outcome (25). Furthermore, the mechanisms that account for the premature elevation of P, despite suppressed endogenous gonadotrophins by GnRH-a, are still unclear. As a pituitary escape from the suppressive effect of GnRH-a is unlikely, serum P elevation could result from exposure to a large amount of exogenous gonadotrophins *(39)* through an increased follicle stimulating hormone (FSH)-induced LH receptivity *(40)*. The contribution of the adrenal gland to both P and androgen production cannot be excluded and this may be reduced by simultaneous dexamethasone administration, but it is still uncertain whether this actually improves the IVF outcome *(41,42)*. At the present time, serum P cut-off levels on the day of hCG, as a means of making a clinical decision with respect to the possible cancellation of the IVF cycle and the cryopreservation of all embryos for future transfer, should be questioned *(43)*.

GnRH antagonists

GnRH antagonists are synthetic analogues of GnRH that compete with endogenous GnRH for pituitary binding sites but are unable to induce GnRH receptor cross-linking, a process that appears to be necessary

to effect calcium ion-mediated gonadotrophin release *(44)*. Clinical advantages of GnRH antagonists over GnRH agonists are the absence of the initial stimulation of gonadotrophin release (flare-up effect) and, as a consequence, a more direct, immediate and reversible suppression of gonadotrophin secretion which allows their use without the need for a desensitization period.

To date, three generations of antagonists have been used. The first- and second-generation compounds exhibited high potency in the suppression of ovulation in rats but induce histamine release, resulting in transient systemic edema and inflammation at the injection site (first generation) or local reactions only (second generation). The third generation of GnRH antagonists has negligible histamine-release properties and comparable anti-ovulatory activity to that of the second generation.

In initial clinical studies, GnRH antagonists were used to prevent a premature LH surge during the menstrual cycle *(45,46)* and, subsequently, during ovarian stimulation for IVF. There are two regimens using GnRH antagonists.

Multiple-dose GnRH antagonist administration

In this protocol, daily injections of low-dose antagonist are given from day six of ovarian stimulation using exogenous gonadotrophins, which is when multifollicular development and estradiol secretion may trigger an endogenous LH surge *(47)*. A multiple dose-finding study performed with orgalutran, one of two available antagonists, clearly demonstrated that the optimal daily dose is 0.25 mg *(48)*. Indeed, this dosage is able to adequately prevent the endogenous LH surge before hCG administration and simultaneously maintain a residual basal LH secretion compatible with a high rate of estradiol secretion, mature oocyte collection and pregnancy. The short half-life of the antagonist with this dose requires a daily administration up to the time of hCG administration *(49)*. A similar study using 0.25 mg cetrotide has confirmed that this dose is adequate to prevent an endogenous LH surge *(50)*.

Single-dose GnRH antagonist administration

The injection of a single and large dose of GnRH antagonist in the late follicular phase has proved to be effective in postponing the spontaneous LH surge in normo-ovulatory women *(46)*. On this basis, several clinical studies have been performed using cetrotide in IVF cycles *(51,52)*. In a comparative study, a 3 mg dose was selected as a safer choice since a "protection period" of at least four days can be obtained *(53)*. When this dose was injected on day eight of the stimulated cycle, or earlier if the ovarian response was more rapid, no LH surge was observed. Moreover, in some cases where plasma LH levels were above 10 IU/L at the time of GnRH antagonist injection, the LH surge was completely blunted *(52,53)*. When ovarian stimulation needed to be prolonged over the three days following the first GnRH antagonist injection, a second large dose or additional daily 0.25 mg doses of the drug were required, due to the relatively short half-life of the antagonist *(53)*. In every situation, oocyte retrieval was performed in the absence of follicular rupture, demonstrating the effectiveness of this regimen in the prevention of the endogenous LH surge.

While the primary goal of GnRH antagonist administration—the prevention of the LH surge—was achieved in these studies, some concern remains about the overall effectiveness of the protocol. Indeed, at least in multicentre studies *(54,55)*, the number of ovarian follicles, collected oocytes and pregnancy rates tended to be lower than those obtained with the long-term GnRH-a protocol. Reasons for these differences in the effectiveness of the two protocols are still poorly understood and may be partly related to the regimen of GnRH antagonist. However, it is likely that other factors, such as the absence of ovarian quiescence before ovarian stimulation, and the regimen of exogenous gonadotrophins, also contribute to the relatively lower effectiveness of GnRH antagonist protocols. Finally, if the results of these studies are adjusted according to each centre, differences between protocol effectiveness were limited, attesting that a learning period is required to adequately control stimulation protocols employing GnRH antagonists. Furthermore, programming the IVF cycle through steroid administration during the cycle preceding IVF may not only be convenient for most centres but could also be effective in improving the size of the cohort of recruited follicles. The benefit of a programming cycle is currently under consideration.

Nevertheless, several advantages have been clearly identified with these protocols. The compliance of patients with the GnRH antagonist protocols was excellent due to the shortened exposure to GnRH analogue administration and to the good clinical

tolerance of this third generation of antagonists. Furthermore, the amount of exogenous gonadotrophins needed for ovarian stimulation was reduced as well as the occurrence of hyperstimulation syndrome *(54,55)*. Finally, the overall cost of this regimen was significantly lower than that of the GnRH-a protocol.

The new GnRH antagonists also permit the design of more gentle stimulation schemes, with the return to the use of clomiphene citrate, minimal stimulation or even natural cycles *(56,57)*.

Gonadotrophins

FSH preparations

Human menopausal gonadotrophin (hMG), extracted from the urine of menopausal women, has been used successfully for many years for ovarian stimulation. It is a mixture of both FSH and LH with low specific activity and also contains other urinary proteins *(58)* which can induce allergic reactions *(59)*.

According to the "two cells–two gonadotrophins" theory, both FSH and LH are required to achieve steroidogenesis. Therefore, for patients whose anovulation is related to hypogonadotrophic hypogonadism, hMG preparations must be used that ensure adequate estradiol production and endometrial maturation for embryo implantation.

However, although LH is necessary for thecal androgen production, the amount of LH required for follicular development and steroidogenesis is minimal. Indeed, when LH secretion is suppressed after long-term GnRH-a administration, FSH alone is sufficient to ensure adequate steroidogenesis and endometrial development. Furthermore, there has been some concern about the theoretical possibility that excessive amounts of LH could compromise successful maturation of the oocyte. Two recent meta-analyses of randomized trials, comparing hMG and FSH for IVF *(60,61)*, concluded that the clinical pregnancy rate per cycle was greater with the use of FSH than with hMG if no pituitary desensitization was undertaken. However, for patients assigned to long or short GnRH-a protocols, the difference between the efficacy of FSH and hMG treatments was less and only significant in the case of FSH *(60)*. These data strongly suggested that the choice of gonadotrophin preparations used for ovarian stimulation during IVF treatment should take into account the GnRH analogue regimen that is used. Finally, a meta-analysis performed in patients with polycystic ovary syndrome (PCOS), showed that the rate of OHSS was significantly reduced when using FSH instead of hMG *(62)*.

The need for an improved product combined with advances in purification techniques led to a highly purified human urinary FSH (u-hFSH), which contains more than 95% pure FSH with a specific activity of 9000 IU FSH/mg. Nevertheless, this preparation still has the inherent disadvantages of all urine-derived preparations which require the collection of large quantities of urine, leading to unreliable supply and, most importantly, batch to batch inconsistency.

Recombinant DNA technologies were used in the early 1990s for the production of recombinant human FSH (r-hFSH) with the insertion of alpha and beta FSH subunits into genetically engineered mammalian cells (Chinese hamster ovary [CHO] cells). The most significant advantage of this technology is that the manufacture of r-hFSH is independent of urine collection, ensuring the consistent availability of biochemically very pure FSH preparation (>99%) with minimal batch to batch variation *(63)*. The purification procedure consistently yields an FSH preparation with a very high specific activity of >10 000 IU FSH/mg and a low level of degradation or oxidation *(64)*.

Two r-hFSH preparations are currently available: follitropin alpha and follitropin beta. While the manufacturing and purification procedures for these two preparations are different *(65)*, it is uncertain to what degree these factors are clinically relevant. There is some evidence that r-hFSH preparations have clinical advantages over u-hFSH or highly purified u-hFSH. Several comparative studies have demonstrated significant advantages for r-hFSH in terms of efficacy as assessed by the number of oocytes retrieved as well as efficiency judged by FSH consumption and the duration of treatment *(66–71)*. Pregnancy rates in individual studies were marginally higher after r-hFSH than after u-hFSH *(72)* when comparable numbers of embryos were transferred. However, when pregnancies after the transfer of frozen embryos were assessed, a statistically significant difference in pregnancy rates was observed in favour of r-hFSH *(73)*. Furthermore, a meta-analysis of 12 randomized trials comparing 1556 patients receiving r-hFSH and 1319 patients receiving u-hFSH in IVF and intracytoplasmic sperm injection (ICSI) programmes *(74)* showed that the pregnancy rate per started cycle was significantly higher with r-hFSH. Thus it may be concluded that clinical practice should favour the use of r-hFSH over urinary preparations.

For these reasons, urinary products have been progressively replaced by r-hFSH preparations in Europe, despite the increased cost. The use of r-hFSH results in a higher pregnancy rate with a lower dose of drug over a shorter period of administration. Furthermore, two recent cost-effectiveness studies in the USA and the UK have shown r-hFSH to be significantly more cost-effective than u-hFSH in terms of ongoing pregnancy *(75)*. Neither social costs nor the costs of patients' time away from work and travel expenses were incorporated into the models. It is assumed that as fewer attempts are required with r-hFSH, the social, employment and travel costs would be lower than with u-hFSH. However, this requires further study.

LH preparations

Following the development of r-hFSH for use in associated reproductive technology (ART), a reliable recombinant DNA LH preparation, free from the potential problems associated with human source material, has been developed. The biological activity of this r-hLH preparation has been demonstrated in the rat seminal vesicle weight gain bioassay and in a primate model of IVF. In both cases, the biological responses to r-hLH and hMG were shown to be similar. Clinical studies were undertaken to investigate both the efficacy and the safety of r-hLH for patients with hypogonadotrophic hypogonadism *(76)*. It was found that daily injections were well tolerated and produced a dose-related effect on estradiol production and endometrial thickness. A daily injection of 75 IU r-hLH seems to be the minimal dose to achieve adequate follicular maturation.

The issue of the need for LH in normal women simultaneously treated with GnRH analogues is still to be addressed. In IVF cycles programmed with a long-term GnRH-a protocol, r-hFSH administration is sufficient to produce adequate folliculogenesis in most cases *(77)*. Preliminary studies have also shown that addition of LH (225 IU) in this situation does not improve the parameters of ovarian stimulation and the cycle outcome *(78)*. However, while it is likely that only a subset of patients would benefit from LH therapy, a study comparing different doses of r-hLH in addition to r-hFSH is needed. No data are available about the use of LH in IVF cycles treated with GnRH antagonists.

Finally, *in vitro* studies have suggested that LH could be involved in the control of follicular growth during the late follicular phase of the cycle *(79)*. Therefore, clinical studies are in progress to evaluate the potential effects of r-hLH in the reduction of the number of developing follicles in hyperstimulated cycles.

Dose of gonadotrophins

There has been no systematic investigation of the optimal dose of FSH needed for controlled ovarian stimulation. A daily dose of 150 IU or 225 IU of urinary or recombinant preparations is usually recommended with subsequent adjustments from day six or seven of stimulation, according to the ovarian response assessed by serum estradiol levels and/or ultrasound. More recently, the introduction of r-hFSH with a higher efficiency than urinary preparations has led to the comparison of several starting doses of FSH in a 100–225 IU range *(80,81)*. Although the design of these studies was not strictly comparable, it seems that 150–200 IU is the standard starting daily dose for patients with no evidence of ovarian dysfunction.

Triggering of ovulation

Human chorionic gonadotrophin

Due to its similarity to LH, urine-derived hCG (u-hCG) has been used clinically in anovulatory women for about 40 years to trigger ovulation and luteinization and to support the corpus luteum. In patients enrolled in IVF protocols which include GnRH-a, u-hCG is used as a surrogate LH surge. Despite the widespread use of u-hCG, commercial preparations suffer from the same disadvantages as other urine-derived gonadotrophins. They require the collection of large quantities of urine from which the extracted starting material is of poor quality, leading to unreliable pharmaceutical activity and unpredictable adverse immunological reactions *(82)*.

The advent of recombinant DNA technology has also permitted the development of a recombinant form of hCG (r-hCG) which is pure, and whose production is independent of urine collection. One product, from genetically engineered CHO cells, is now commercially available and preliminary studies have estimated that 250 μg r-hCG correspond to 5000 IU u-hCG. Two recent studies comparing u-hCG and r-hCG in women undergoing ovulation induction for ART, concluded that the final oocyte maturation was similar *(83)* or

better *(84)* following the administration of the recombinant preparations. Moreover, in both studies, serum P concentrations on day one and days six and seven post-hCG, and serum hCG concentrations at all post-hCG time points, were statistically higher in the group treated with r-hCG preparations. Furthermore, the incidence of adverse events was significantly higher in the u-hCG group while the incidence of injection-site reactions was significantly lower in the r-hCG group. Therefore, r-hCG seems to have significant advantages compared to u-hCG.

Recombinant LH

Although human LH produced by DNA recombinant technology has similar pharmacokinetic characteristics to the pituitary-derived hLH, r-hLH has a terminal half-life of about ten hours compared to the 30 hours of u-hCG *(85)*. This difference between the two preparations may be important in the prevention of OHSS which is likely linked to the long half-life of u-hCG.

In humans, r-hLH has been tested for triggering final follicular maturation before IVF. The first pregnancy using this approach was reported in 1996 *(86)*. More recently, a large multicentre, double-blind study has been undertaken to compare different doses of r-hLH in 258 patients enrolled in a regular IVF protocol following pituitary desensitization with GnRH-a. Each dose of r-hLH, from 5000 to 30 000 IU, appeared to induce an adequate final follicular maturation with a percentage of mature and fertilized oocytes similar to that found with u-hCG. However, a second dose of 15 000 IU of r-hCG, two days after the first injection, appeared to be required to adequately support the luteal phase. As this observation was made in patients whose low residual secretion of endogenous LH was induced by pituitary desensitization, it deserves confirmation in cycles without desensitization with treatment protocols including GnRH antagonists. This study also confirmed that the duration of the exposure to LH/hCG is a major determinant for the development of OHSS.

GnRH agonist

The endogenous LH surge induced by GnRH-a works through an indirect mechanism that relies on the patient's own pituitary response to GnRH. The GnRH-a induced LH surge has a sharper profile with a higher peak value but a shorter duration than the natural LH surge *(87)*. Consequently, in clinical practice, triggering ovulation with GnRH-a may be considered in women at risk of OHSS or multiple pregnancy and in cycles where pituitary desensitization has not been previously performed. The efficacy and safety of the LH surge induced by GnRH-a was first investigated in WHO Group II anovulatory PCOS patients *(87–90)*. These studies showed that the risks of OHSS and multiple births were not totally blunted and demonstrated a high incidence of luteal phase deficiency. Therefore, luteal phase support is likely to be required when this ovulation triggering regimen is applied. Furthermore, in normally ovulating patients whose ovarian stimulation was performed before IUI, GnRH-a administration was reported to improve the pregnancy rate and to abolish the risk of OHSS *(91)*. Finally, preliminary results have been published in patients whose endogenous LH surge was prevented by GnRH-a administration *(92)*. An LH surge was successfully elicited by administration of triptorelin (one bolus of 0.1 mg) but no pregnancy was reported. Altogether, these studies show that triggering ovulation through GnRH-a administration may be useful for patients or cycles at risk of OHSS and multiple pregnancy, but a further evaluation of the outcome of this treatment is needed.

Luteal phase support

Progesterone (P) and estradiol (E_2) play central roles in the maintenance of human reproduction. Steroid production peaks about four days after ovulation and continues at this level for about a week, falling about five days before the next menstrual period *(93)*. During this time, P is secreted in a pulsatile fashion and P production is 40-fold the maximal E_2 production. Until the luteo-placental shift occurs at about seven weeks of gestational age, the ovary's production of these hormones is critical to pregnancy maintenance *(94)*.

In stimulated cycles, the luteal phase differs from the natural one because hormone production from multiple corpora lutea is supraphysiological. Moreover, the luteal phase may be shortened in relation to a sharper decline in serum P and E_2 than in natural cycles. For this reason, the principle of luteal support was widely adopted and this policy was further reinforced with the advent of GnRH-a use in the late 1980s *(95)*. Nevertheless, the supraphysiological steroid levels following ovarian stimulation may also

have adverse effects on uterine receptivity, even when luteal length is adequate. Indeed, advanced endometrial maturation and increased uterine contractions have been observed in high-responding women *(96–97)* with a risk of a lower pregnancy rate *(98)*.

In stimulated IVF cycles, steroid production during the first week after ovum retrieval is likely to be well-timed and sufficient. Therefore, the start of exogenous steroid support does not seem to be critical. In contrast, the timing of P administration is critical in ovum donation programmes where the only source of P is exogenous. The highest pregnancy rates are likely to occur when two-day-old embryos are transferred on the fourth or fifth day of P therapy *(99)*.

While P alone is recommended in a conventional luteal support regimen, E_2 supplementation should also be considered. Indeed, even if E_2 does not directly mediate luteinization, it may be required to stimulate P receptor replenishment. For this reason, hCG administration has been advocated for its stimulating effect on E_2 and P secretion by the corpora lutea. Meta-analysis has shown that hCG injections seem to be more effective than P alone in terms of pregnancy rates in GnRH-a cycles *(100)*. However, the increased risk of OHSS following repeated hCG administration during the luteal phase may explain why the use of hCG has not been widely adopted. Furthermore, if the superiority of hCG over P is related to its ability to stimulate E_2 production, adding exogenous E_2 to P supplementation must be considered as a safer alternative. This issue has been recently addressed in a study performed in high-responding patients treated with a long-term GnRH-a protocol *(101)*. Patients who received both E_2 and P had higher implantation rates and lower spontaneous abortion rates than those who did not. Therefore, luteal supplementation with both steroids may be a better alternative than the use of repeated hCG injections, at least in patients at risk of OHSS.

Several routes of P delivery have been proposed, including oral, intramuscular and transvaginal.

Although the development of micronized formulations of natural P has resulted in preparations with improved absorption and bioavailability, the systemic P levels that can be achieved with these preparations following oral ingestion (100 mg) are too low to provide adequate endometrial support *(102)*. This may be related to a first-pass effect following oral administration of P and its extensive hepatic metabolic degradation *(103)*. Several clinical trials of oral supplementation with natural P in IVF cycles (200 mg three times daily) have confirmed the inadequacy of this route compared with the other routes of administration *(104–106)*.

Intramuscular administration of P significantly increases its bioavailability *(107)*. The usual intramuscular dose varies from 25 mg to 100 mg daily, sometimes in divided doses. Serum peak levels are well above the physiological range and endometrial maturation is "in phase"; this is associated with good clinical results *(108)*. Furthermore, a comparison of oral micronized P with intramuscular P resulted in significantly higher implantation rates with the latter treatment *(105)*. However, the intramuscular route also has several drawbacks: it is inconvenient, uncomfortable for the patient and it may produce some local side-effects, such as marked inflammation at the injection site.

The vaginal route offers several important advantages over intramuscular dosing as it avoids the first-pass hepatic metabolism and ensures sustained plasma P concentrations. Progesterone absorption is further influenced by the formulation used (tablets, suppositories, creams, oil-based solutions or, more recently, slow-release polycarbophil gel) *(109)*. Compared with intramuscular P, higher doses of vaginal P are often necessary to achieve adequate serum P levels but, using sustained-release formulations, lower doses (45–90 mg) applied once a day or even once every other day, might be effective *(104,110–111)*. As shown by endometrial biopsies performed in the midluteal phase, most endometria were in phase after the use of vaginal micronized P *(112)*, even though serum P levels were less than normal *(113)*. This suggests that vaginally administered P exerts a pronounced local effect on the endometrium, the so-called "first uterine pass effect". While comparison between vaginal and intramuscular P administration provided contradictory results *(109)*, a recent study in a donor ovum programme showed an improvement in the cycle outcome with the use of sustained-release formulations *(114)*. The vaginal route has proved to be a valuable route for drug delivery in infertility treatment.

Monitoring of treatment

The main objectives of monitoring treated cycles are to control follicular maturation, to time hCG administration and to predict the outcome of the cycle. Another purpose of ovarian monitoring is to prevent

OHSS by cancelling cycles at risk for high ovarian response or, alternatively, to detect poor ovarian response and adjust ovarian stimulation accordingly. This approach is valid for an IVF cycle and, to a lesser extent, for programming an IUI cycle.

The combination of serum E_2 determination and ultrasonography has long been accepted as the most widely used mode of follow-up during COH.

In the early development of IVF when GnRH analogues were not available, clinical studies emphasized the need for monitoring serum E_2 concentrations and different serum E_2 patterns were correlated with cycle outcomes *(115,116)*. However, the level of serum E_2, although a functional indicator of folliculogenesis, is not always correlated with follicular growth *(117)*. Due to the considerable variety of protocols used in ART cycles, no description of a common and optimal E_2 pattern is available. Nevertheless, there is some evidence that, whatever the protocol used, a plateau of plasma E_2 values for more than three days is associated with a poor outcome of the ART cycle. Conversely, measurements of plasma E_2 are helpful in predicting excessive ovarian response and in deciding subsequent doses of gonadotrophin or the cancellation of the cycle or the ET.

Serum E_2 levels were used as an early marker of ovarian responsiveness to exogenous gonadotrophins. On the fourth day of treatment, assessment of serum E_2 levels may predict the subsequent ovarian response to exogenous gonadotrophins *(118)*. Similarly, evaluation of E_2 response to the endogenous gonadotrophin flare-up induced by GnRH-a in the short-term protocol, was designed as a "lupron screening test"*(119,120)*. Both the E_2 pattern and the maximal E_2 response following the first injections of GnRH-a have been correlated with the subsequent ovarian response to COH *(121)*. These data underline that a single determination of plasma E_2 may be a helpful predictor of a poor or high ovarian response and useful for the tailoring of gonadotrophin administration.

Determination of plasma E_2 is also recommended for the assessment of whether or not pituitary desensitization induced with a long-term GnRH-a protocol is effective at the ovarian level. Indeed, as plasma LH immunometric evaluation may not adequately reflect the state of pituitary desensitization, it is commonly stated that plasma E_2 must be lower than 180 nmol/ml to ensure that ovarian activity is actually suppressed. In every situation, it is recommended that ovarian stimulation is started with FSH only when ovarian activity has been suppressed, whatever the duration of GnRH-a administration required to obtain suppression of ovarian activity.

Finally, the recent availability of GnRH antagonists in ART cycles provides the opportunity of reconsidering the role of plasma E_2 determinations during the stimulation phase of the ART cycle. Indeed, administration of a GnRH antagonist may alter the pattern of plasma E_2 response *(54)*. However, it is still not clear if a decrease in serum E_2 levels after GnRH antagonist injection is responsible for the lower pregnancy rate sometimes observed with this protocol. Plasma E_2 determination is a valuable tool for monitoring ART cycle treatment while the value of plasma LH measurement seems to be strictly limited to cycles stimulated without the addition of GnRH analogues.

Additionally, ultrasound measurement of follicular growth plays a key role in the assessment of the adequacy of follicular maturation and of the correct timing of hCG administration. In most clinical studies, triggering of ovulation by hCG is recommended when at least three large (>16 mm) follicles have been visualized on ultrasound. Furthermore, measurement of endometrial thickness through a vaginal probe allows an indirect assessment of E_2 secretion. With the extensive use of GnRH-a protocols, it has been emphasized that patient follow-up could be simplified by using only ultrasound determination of both follicular growth and endometrial maturation and this approach seems effective in triggering ovulation without reducing the pregnancy rate *(122)*. This minimal monitoring is more cost-effective and especially relevant in low-resource settings.

Clinical indications for ART

Intrauterine insemination

The rationale for performing IUI is to increase the number of highly motile spermatozoa with a high proportion of normal forms at the site of fertilization. While in the past, the whole ejaculate was placed in the uterus, new semen preparation techniques derived from IVF procedures and able to remove prostaglandins, bacteria and immunocompetent cells, have led to the recommendation that a direct transfer of motile spermatozoa after sperm preparation and concentration in a small volume of medium is to be preferred. Several studies have shown that IUI is more effective than intravaginal or intracervical insemina-

tions with unprepared semen *(123)*.

Indications for IUI include:

- physiological and psychological dysfunctions, such as hypospadias, vaginismus, retrograde ejaculation and poor erectile function;
- cervical hostility related to poor quality mucus or persistently negative postcoital tests;
- male infertility: a minimal amount of total motile sperm cells (at least one million) should be inseminated for optimal results *(124)* and it is presumed that a certain degree of normal morphology of spermatozoa is also required to achieve pregnancy. Treatment should be restricted to IUI alone, because induction of ovulation has little additional beneficial effect *(125)*;
- unexplained infertility in both the male and the female partner.

While both IUI and COH independently increase the probability of conception *(126,127)*, COH seems to be a more contributory factor than IUI by overcoming some subtle cycle disorders. In male and in unexplained infertility, it has been recommended that three to six cycles of IUI are undertaken and, if unsucessful, the couple can be offered IVF/ICSI.

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection is primarily indicated in the most severe forms of male infertility with very low sperm count, poor motility and/or high teratospermia, and in males with obstructive or idiopathic azoospermia by using epididymal or testicular sperm. ICSI is also currently used in couples who have failed to provide an embryo through routine IVF procedures. These topics are discussed elsewhere in this volume.

Eligibility of patients

Determinants of ovarian response

Age

The age of the female partner is the single most important factor determining spontaneous fertility and the effect of age is enhanced when considering the outcome of all forms of fertility treatment. Age is a particularily strong factor when the outcome of ART treatment is considered *(128,129)*. As reported in many countries *(130,131)*, pregnancy rates are fairly constant (at around 25% live births per cycle) up to the age of 34 years, when there is a steep decline. No pregnancies have been recorded among women over the age of 46 years. While many factors may contribute to the deleterious effect of age, there is compelling evidence that ovarian function is altered. Not only is the number of follicles available decreased with advancing age but oocyte quality is reduced and the incidence of aneuploidy increased. Therefore, the ageing ovary is a major concern in infertility treatment, particularly when COH must be achieved. The assessment of ovarian function is a key issue before performing COH.

Duration of infertility

While the duration of infertility is a major factor determining the likelihood of spontaneous pregnancy occurring in untreated infertile patients *(132)*, it is still uncertain whether reduced effectiveness of ovarian stimulation is related to the duration of infertility or to the age of the woman, which are clearly closely linked.

Weight

Obesity is often associated with menstrual disorders and anovulation *(133)*. As the impact of weight gain on the reproductive system is multifactorial, the respective roles of abnormalities such as hyperandrogenism, hyperinsulinism or estrogen production from adipose tissue is still a matter of debate. Furthermore, obesity itself may be a factor affecting ovarian response. Indeed, in women with no ultrasound evidence of PCOS, a higher dose of clomiphene citrate is needed to obtain ovulation *(134)* and the response to gonadotrophin induction of ovulation is inversely related to body mass index *(135)*. Moreover, PCOS patients with moderate obesity have a blunted response to gonadotrophin therapy compared with their nonobese counterparts *(136)* and require a more prolonged stimulation and a higher dosage of FSH *(137)*. It is likely that the pharmacokinetic profile of ART drugs is different in overweight compared to lean patients and may partly explain the need for higher doses. Weight reduction is beneficial in restoring ovulation or in reducing drug dosage. Weight reduction in obese patients reduces hyperandrogenism and hyperinsulinaemia, both factors influ-

encing the ovarian response to FSH. Therefore, it is presumed that obesity is responsible for the relative ovarian insensitivity to infertility treatment.

Smoking

Epidemiological studies have shown that 38% of nonsmokers conceive in their first ART cycle compared with 28% of smokers, with smokers being 3.4-fold more likely to take more than one year to conceive than nonsmokers *(138)*. Both active and passive smoking have been associated with elevated FSH concentration *(139)*. It has been postulated that impaired fertility and delayed conception among women who smoke result from interference with gametogenesis or fertilization, failure of implantation and early miscarriage *(138)*. As far as ovarian function is concerned, some studies have shown that smoking women, in particular, young women, had a higher basal and post-clomiphene citrate test serum FSH *(139–141)*. Other studies have shown a detrimental association between cigarette smoking and ovarian response to stimulation. Women who smoke required a significantly higher dosage of gonadotrophins than nonsmokers *(141–143)*. Hence smoking may further increase the cost of ovarian stimulation and this must be emphasized to the infertile couples in whom the female partner is a smoker.

Management of poor ovarian responsiveness

Diminished or poor ovarian response to COH occurs in about 9%–24% of patients *(144)* and is still a challenging issue. One of the main reasons may be related to the lack of a universally accepted definition of "poor responders".

The original definition of poor responders was based on low peak E_2 concentrations (<1000 pmol/ml) during ovarian stimulation with 150 IU hMG *(145)*. The low pregnancy rate in these patients was attributed to the low number of recruited follicles and retrieved oocytes. Later, the definition evolved with the advent of more aggressive stimulation protocols to peak serum E_2 values greater than 1700 pmol/ml and less than four dominant follicles on the day of hCG administration.

Another approach involved patients without previous experience of stimulatory ART cycles but whose diminished ovarian reserve could predict a poor response to gonadotrophins. Indeed, several tests of the functional ovarian reserves seem reliable for predicting a low response to standard protocols *(146,147)*.

These tests include:

- day three determination of basal serum FSH *(148–150)*, E_2 *(151,152)*, inhibin B *(153)* or the FSH/LH ratio *(154)*;
- dynamic tests, such as the clomiphene citrate challenge test *(155,156)*, the GnRH-a stimulation test *(119,120,157)* and the exogenous FSH ovarian reserve test *(158)*;
- imaging techniques, such as measurement of ovarian volume *(159,160)*, antral follicle count *(161,162)*, and measurement of ovarian stromal bood flow with colour Doppler *(163,164)*.

Several stimulation protocols have been proposed to improve the outcome in poor responders. These include:

- varying the dose or the day of the cycle for initiating stimulation with gonadotrophins *(165–169)*;
- pituitary desensitization with a GnRH-a long protocol followed by stimulation with a high dose of gonadotrophins *(170)*;
- initiating GnRH-a and gonadotrophins together in a short-term protocol *(171,172)*;
- cotreatment with growth hormone or growth hormone-releasing hormone *(173–176)*;
- cotreatment with estrogens or combination oral contraceptives *(177,178)*;
- using clomiphene citrate for stimulation *(179, 180)*;
- natural IVF cycles *(181)*.

However, while some of these protocols may improve the ovarian response to stimulation, none of them were able to significantly improve the pregnancy rate. Other approaches have been shown to be more successful in improving the outcome of IVF cycles. All recommend the reduction of GnRH-a or manipulation of the agonist differently, for example:

- a "micro-dose" GnRH-a flare-up regimen: after a pretreatment with oral contraceptives, patients received microdose of GnRH-a (20–40 μg of leuprolide twice daily) in the early follicular phase and started gonadotrophins on the third day *(182–184)*;

- reducing the dose of GnRH-a when ovarian desensitization is achieved has been proposed as a "mini-dose" long protocol *(185)*. Dosage of GnRH-a may be reduced by half or a fifth without risk of a premature LH surge *(186)*;
- the "stop-lupron" protocol which involves stopping GnRH-a administration with the onset of menses and stimulation with high-dose gonadotrophin therapy *(187, 188)*.

With these protocols, some advantage was observed in terms of pregnancy and it is possible that they may be an effective approach in those cases in which the use of GnRH-a is presumed to be responsible for the poor ovarian response to gonadotrophins.

From these data, it is clear that the different causes of poor ovarian response should be taken into consideration when deciding what protocol is the most suitable and in what circumstances the cycle must be cancelled. As an example, in young, poor-responder patients with normal serum FSH basal values, it has been reported that a low response to gonadotrophins does not adversely affect the IVF cycle outcome *(189, 190)*. Conversely, the least favourable situation is clearly that of patients with a low ovarian reserve in relation to their advanced age, or ovarian dysfunction. They do not benefit from the use of ICSI *(191)* and should be considered as candidates for oocyte donation.

Management of PCOS

This is one of the more common endocrine disorders with a very heterogeneous definition, from the single finding of polycystic ovarian morphology detected by ultrasonography to a complete form with clinical symptoms such as obesity, hyperandrogeny, cycle disorders and infertility *(192)*. It is estimated to be the major cause of anovulatory infertility (accounting for 73% of cases) and hirsutism *(193)*. There is evidence that ovarian hyperandrogenism is the main factor responsible for persistent anovulation *(194)* and it is likely that the hyperinsulinaemia usually observed in these patients may contribute to the infertility.

The strategy for ovulation induction in PCOS anovulatory women must take into account that the risks of hyperstimulation and multiple pregnancy are markedly enhanced with gonadotrophin therapy. Therefore, clomiphene citrate administration has been recommended as the first-line therapy in these patients. Indeed, clomiphene citrate induces ovulation in about 70%–85% of patients although only 40%–50% conceive *(195)*. While a large range of daily doses has been studied, it must be emphasized that an exuberant response may be observed with 50 mg in some patients and, in the USA, the maximal dose approved by the FDA is 100 mg/day for five days. The use of clomiphene citrate is currently recommended for only six months because of the putative increased risk of ovarian cancer.

Gonadotrophin therapy is indicated for women with anovulatory PCOS who have been treated with clomiphene if they have either failed to ovulate or have a response to clomiphene that is likely to reduce their chance of conception (for example, negative post-coital tests). To prevent the risks of overstimulation and multiple pregnancy, traditional protocols have been replaced by either low-dose step-up, step-down regimens or by a sequential step-up, step-down protocol. Several studies have shown that these regimens are effective and safe by reducing the number of leading and medium-sized follicles *(196–198)*. The risks of multiple pregnancy and OHSS are further reduced if ovulation is triggered with a single injection of hCG and in the absence of more than two follicles larger than 16 mm or more than four follicles larger than 14 mm. In overstimulated cycles, hCG is withheld and the patient advised to refrain from unprotected sexual intercourse. Many published series support the notion that carefully conducted ovulation induction therapy results in a good cumulative conception rate in women with PCOS *(199)*.

Different gonadotrophin preparations have been used to induce ovulation in PCOS anovulatory women. Two meta-analyses were carried out on the use of FSH versus hMG treatment. The first concluded that FSH is associated with a reduction of moderate-to-severe OHSS *(62)*. The other *(60)* showed that treatment with FSH results in a 50% higher pregnancy rate in IVF cycles. In spite of the well-known drawbacks of meta-analyses, it may be concluded that FSH is more suitable in PCOS patients to reduce the risk of OHSS, which is still a major concern with this therapy.

As hypersecretion of LH is a classical endocrine feature of PCOS and may result in reducing the conception rate *(200)*, particular attention has been paid to the possible advantages of adding GnRH-a prior to ovarian stimulation. However, prospective randomized studies have indicated that GnRH-a provide no benefit over gonadotrophin alone and do

not reduce the tendency to multifollicular development, cyst formation and OHSS *(201,202)*. For patients resistant to ovulation regimens, IVF therapy has been advocated as another modality but the persistent risk of OHSS justifies recommending careful administration of FSH.

Finally, reducing hyperinsulinaemia through the administration of insulin-sensitizing agents such as metformin, is effective in decreasing serum androgens in both obese and nonobese PCOS patients *(203)*, improving spontaneous ovulation *(204)* and the ovarian responsiveness to clomiphene citrate in obese PCOS patients *(205)*.

Risks of COH

The side-effects of COH still remain the challenging issue of ovulation induction. Some of them are more serious, OHSS being the most serious complication. Others are longer-term side-effects with a special concern regarding the risks of neoplasia and the morbidity and mortality associated with multiple births.

OHSS

The worldwide incidence of severe OHSS has been estimated at 0.2%–1% of all ART cycles *(206)* and the associated mortality at 1:45 000–1:50 000 per infertile women receiving gonadotrophins *(207)*. However, the frequency of this life-threathening situation depends on many factors such as criteria used for diagnosis, identification of patients at risk, the type of medication and the use of preventive measures. For example, after IVF, the overall incidence is reported to be 0.6%–14% *(208)*. Thus, clinicians have to balance the risks and benefits of medical intervention when considering treatment options.

While the pathogenesis of OHSS has not been completely elucidated, it is likely that the increased capillary permeability triggered by the release of ovarian vasoactive substances under hCG stimulation plays a key role in this syndrome. Factors belonging to the renin–angiotensin system, cytokines and vascular endothelial growth factor (VEGF) are also involved in this process *(209)* and awareness of these mechanisms may provide opportunities for the design of specific treatment regimens. While there is no consensus about the management of OHSS, it is agreed that prevention of this syndrome is the main objective.

Individualizing ovulation induction protocols may lead to better control of ovarian hyperstimulation. Several factors may be taken into account including a history of exaggerated response to gonadotrophins in previous cycles and ultrasonographic appearance of PCOS.

Using step-wise regimens and, if needed, early cancellation based upon serum E_2 and ultrasound findings, withholding hCG administration or cancelling oocyte pick-up have been proven to be efficient in reducing the occurrence of OHSS. However, these approaches take a heavy emotional and financial toll on patients and do not resolve the issue of the infertility. Therefore, other less drastic prevention measures have been proposed such as "coasting".

Coasting consists of stopping gonadotrophin stimulation for one or more days and seems to be a valuable method to reduce the incidence and severity of OHSS in patients at risk *(210–212)* without compromising the cycle outcome *(213,214)*.

As the incidence and duration of severe OHSS is greatest for patients who conceive *(215)*, cryopreservation of all embryos has been proposed as an alternative way to minimize hCG exposure without cancelling oocyte retrieval *(216)*. Subsequent transfer of cryopreserved-thawed embryos in a programmed cycle with steroid substitution yields a good pregnancy rate *(217)*. However, as the obstetric outcome of pregnancies complicated by severe OHSS could be worsened *(218)*, interruption of pregnancy when life-threatening situations occur may be need to be considered.

An attempt to distinguish between early OHSS (three to seven days after hCG) related to high estrogen levels and late OHSS (12–17 days after hCG) associated with clinical pregnancy could help to better define the preventive effects of these therapeutic approaches *(219)*.

Neoplasia

Breast and ovarian neoplasia are of multifactorial etiology and infertility is one of the factors considered to increase the risk. A linkage between the drugs used in ART and breast or ovarian cancers has not yet been fully established *(220)*.

Ovarian cancer

Ovarian cancer represents the sixth most common

cancer in women and is the most fatal gynaecological malignancy with a 5-year survival rate of about 40% *(221)*. However, the incidence of ovarian cancer varies widely among countries *(222)* and a large number of identifiable factors have been associated with increasing risk of ovarian cancer, including environmental, hormonal and genetic factors. Parity and oral contraceptive use have well-documented protective effects as does tubal ligation and hysterectomy. The explanation for these protective effects possibly involves decreased ovulation, at least in older infertile women *(223)*. Conversely, infertility and "incessant ovulation" have been documented as risk factors for the development of ovarian cancer *(224)*. As a result of this consistent association, it has been hypothesized that the hyperstimulatory effects of fertility medication may be linked to the genesis of some cases of ovarian cancer. However, inability to conceive is by itself a risk factor for ovarian cancer independent of nulliparity *(225)*. Therefore, the fundamental question is whether the use of drugs for the induction of ovulation independently increases a woman's risk of ovarian cancer over and above that predicated by infertility alone or infertility in conjunction with low parity.

After the first report in 1971 of a possible relationship between incessant ovulation and ovarian cancer *(226)*, it was suggested that epithelial inclusion cysts in the ovarian surface epithelium, which occur in association with ovulation, may be the source of such neoplasms. The first case of invasive epithelial ovarian cancer associated with ovulation induction was described in 1982 *(227)* and was followed by several additional case reports, cohort and case–control studies *(228)*. Concern about the risk of ovarian cancer and the use of fertility medications was recently highlighted in a series of publications *(229, 230)* which reported that infertile women using fertility drugs are three times more at risk for invasive epithelial ovarian cancer than women without a history of infertility. Conversely, in these studies, infertile women not using fertility drugs were reported to have no increased risk. However, critics of these reports have cited selection bias, wide confidence intervals, lack of a uniform etiology of infertility and temporal incompatibility between licensing of modern fertility drugs and treatment for infertility in the subjects in these studies *(231)*. In addition, no attempt was made to control for confounding factors such as infertility itself or family history of ovarian cancer.

In a well-designed and executed large case–cohort study *(232)*, infertile women using clomiphene citrate had a threefold increased risk of developing any ovarian neoplasm but, when infertile clomiphene citrate users were compared with infertile nonusers, no statistically significant increased risk was observed. Nevertheless, the use of clomiphene citrate for more than 12 ovulatory cycles was associated with an increased risk of developing an ovarian neoplasm. However, if the relationship between clomiphene citrate use and ovarian cancer had been truly causal, a more evident dose–response relationship would have been expected.

The possibility of a relationship between infertility secondary to underlying ovarian pathology and ovarian cancer has been also suggested *(233)*. It is also possible that follicular hyperstimulation may drive an already existing epithelial ovarian neoplasm to become clinically apparent. However, evidence against this hypothesis is provided by other studies that have shown epithelial ovarian carcinomas to be insensitive to gonadotrophins *(234)*.

Finally, IVF procedures including not only ovarian hyperstimulation but also repeated minor trauma for ovum pick-up, were associated with some cases of ovarian cancer. However, comparison between stimulated and natural cycles for IVF in women could not find any excess risk for ovarian cancer in the treated group *(235)*. By contrast, a significant association was detected between a diagnosis of unexplained infertility and invasive epithelial ovarian cancer.

Breast cancer

Breast cancer is the leading cancer in women and the use of oral contraceptives and hormone replacement therapy as risk factors remain controversial. Many studies have failed to observe excess risk for breast cancer among infertile women *(236)*. Furthermore, no excess risk for breast cancer can be attributed to ovulation induction. On the contrary, some antiestrogenic agents, such as clomiphene citrate, could be considered protective because of their similarity to tamoxifen *(237)*. However, a diagnosis of breast cancer during or shortly after infertility treatment should warrant close medical follow-up.

Multiple births

The medical, social and financial risks caused by multiple birth needs to be addressed in terms of policy and practice. Iatrogenic multiple pregnancy occurs

after ovulation induction with clomiphene citrate and gonadotrophins, with reported incidence figures of 5%–10% and 16%–40%, respectively *(238)*. The most common result is twinning but the greatest relative increase consists in triplet and quadruplet pregnancies. ART has affected the rate of multiple births in two ways: first, the procedures themselves have a direct impact on the incidence of multiple pregnancy; second, the number of couples undergoing infertility treatment has increased dramatically. As far as the procedures are concerned, it is obvious that the use of more aggressive ovarian stimulation for IVF has resulted in more oocytes being collected and a greater number of embryos being transferred. Nevertheless, recent adoption by several countries of a restrictive transfer policy has led to a significant reduction in high-order multiple pregnancies *(239)*.

Paradoxically, some major concern remains regarding the policy of ovulation induction for chronic anovulation or in preparation for IUI. Indeed, as the results of IUI from natural cycles proved to be inferior to those from gonadotrophin-stimulated cycles *(240)*, ovulation induction is currently performed in normally ovulating patients. As a consequence, multiple pregnancies after ovulation induction alone or associated with IUI, have accounted for the majority of all multiple pregnancies related to infertility treatment *(241)*. This is partly due to the development of a large number of leading follicles (more than two or three) *(242)*. However, careful monitoring of stimulated cycles can reduce, but not totally eliminate, this risk. Indeed, according to an extensive retrospective study *(243)*, the peak serum E_2 concentration and the total number of follicles are independent predictors of the risk of high-order multiple pregnancy. However, as recently shown *(244)*, the number of follicles with a diameter of 12 mm or more seems to be more predictive of multiple birth than that of mature (>16 mm) follicles. Therefore, the current guidelines for ovulation induction, based upon the number of large-sized follicles, may be inadequate for reducing the incidence of high-order multiple pregnancies. Nevertheless, in clinical practice, the best way to minimize the risk of multiple pregnancies is to use much milder stimulation regimens, to carefully control follicular development with both hormonal and ultrasound assessments, and to cancel the cycle in cases of overstimulation. The ovarian stimulation policy must also take into account other parameters such as the patient's age, duration of infertility, previous parity and etiology of infertility, all factors that interfere with the potential risk of multiple pregnancy following induction of ovulation *(245)*. Development of new ovarian imaging technologies may be helpful in improving the reliability of ovarian monitoring. This strategy could limit the use of IVF procedures as an alternative way to avoid the risk of high-order multiple pregnancy *(243)*.

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